ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.

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Pol Herrero Gil

DOCTORAL THESIS

Supervised by

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Departament de Química Analítica i Química Orgànica

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DEPARTAMENT DE QUÍMICA ANALÍTICA I QUÍMICA ORGÀNICA

La Dra. ROSA MARIA MARCÉ i RECASENS, Catedràtica del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. EVA POCURULL i AIXALÀ, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

FEM CONSTAR:

Que la present Tesi Doctoral, que porta per títol: "ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE", presentada per POL HERRERO GIL per optar al grau de Doctor per la Universitat Rovira i Virgili amb menció europea, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, tots els resultats presentats són fruit d'experiències realitzades per l'esmentat doctorand, i compleix els requeriments per a poder optar la menció europea.

I, per a que consti, expedim aquest certificat a Tarragona, 18 de juny de 2014.

Dra. Rosa Maria Marcé i Recasens

Dra. Eva Pocurull i Aixalà

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Quan arriba el moment d'escriure aquestes línies significa que allò que va començar fa quatre anys ençà arriba a la seva fi. És el moment de tancar els ulls i recordar tots els moments viscuts aquests darrers anys, i poder així, recordar a tothom que n'ha format part i poder dir-los senzillament, però sincera i sentida, moltes gràcies.

Les meves primeres paraules són per la Dra. Rosa Maria Marcé. Va ser ella qui va respondre el meu correu electrònic on preguntava per la possibilitat d'optar a una beca predoctoral i sense la qual, res del que ve a continuació hagués tingut lloc. Ella ha resultat ser la meva directora de Tesi juntament amb la Dra. Eva Pocurull i són les que han fet que tot això cobrés sentit. És per això que vull expressar el més sentit dels meus agraïments vers elles, tant per haver-me guiat durant aquest anys, com per la bondat com a persones que m'han mostrat cada dia que he estat al seu costat.

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Ara és el moment de donar les gràcies a tota aquella gent que ha estat al meu costat en el dia a dia al laboratori al llarg d'aquests quatre anys. De veritat, moltes gràcies a tots pels moments viscuts tant a dins com a fora del laboratori, per ajudar-me quan ho he necessitat i per fer que venir cada dia al laboratori fos un regal. Gràcies de tot cor Anna, Alejandro, Antonio, Carol, Cristian, Daniela, Desireé, Henry, Igor, Irene, Julio, Laura T, Laura V, Marta Palomo, Marta Pedrouzo, Mireia N, Montse, Núria G, Núria M, Noelia, Sameer, Silvia E, Sílvia M i Tatiana. També, des d'aquestes línies vull recordar a un amic que malauradament ens va deixar fa un temps i tan sols voldria dir-te, que allà on siguis, ha estat un plaer poder-te conèixer. No t'oblidarem, Maarten.

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CHAPTER 1. INTRODUCTION

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Modern society is intrinsically linked to the extensive use of chemical substances, either to improve the quality of life or achieve more efficient and economically suitable industrial processes. Over the last few centuries, many chemicals have been produced and used in unregulated way, which has resulted in the contamination of the surrounding environment. As an example of the number of chemicals currently used in the European Union, more than 100,000 chemicals are registered and their use is estimated to be around 300 million tonnes per year [1]. Thus, it is obvious that these large amounts of chemical substances may result in an unhealthy impact on the environment and living species. This is shown in a report by the World Health Organization (WHO), which estimates that a significant number of human diseases are caused as a result of prolonged exposure to environmental pollutants [2].

Evidence of environmental pollution has existed since the 1800s but knowledge of environmental pollution has been growing significantly over last few decades due to the interest of the scientific community and government agencies in public and environmental health. At the same time, technical advances in analytical chemistry in terms of the development of more sensitive and selective techniques have helped the scientific community to ascertain that lots of chemical compounds are present in environmental matrices. Examples of this are pharmaceuticals, synthetic fragrances, detergents, disinfectants, plasticisers and preservatives, among others. Some of these compounds have been used for a long time but have only recently been detected in environmental matrices because they are present at parts per billion or per trillion [3]. All of these compounds are known as emerging organic contaminants (EOCs), a term which refers to chemical substances whose continuous emission into the environment may be hazardous for the recipient ecosystems and mankind. Preliminary studies on emerging organic contaminants were performed in the 70s and 80s but systematic research in this field started in the 90s [4,5].

Nowadays, one of the environmental research areas that has received most attention from the scientific community is water chemistry, which includes its quality, safety and treatments for its reuse and purification. Water safety and quality are strongly linked to the contaminants present in it. Thus, environmental agencies have regulated different contaminants which are already known or suspected to be hazardous for human health in public water supplies. For example, the United States Protection Agency (USEPA) regulates more than 90 contaminants in drinking water [6]. As well as the determination of regulated compounds, monitoring studies of non-regulated compounds are necessary to determine which substances should be regulated in the future. These studies are not limited to drinking water analysis but

rather they are also conducted on surface and ground water, as these are the main sources for drinking water supplies. Moreover, studies on sewage are also very frequent in order to estimate the capabilities of sewage treatment plants to provide safe treated water for its management and reuse. As a result of these studies, new emerging organic contaminants are still being discovered and, in general, there is an overall lack of information of the ecotoxicological impact for most of these EOCs in terms of their toxicity, bioaccumulation and occurrence in different environmental compartments and species.

As mentioned above, the extensive use of several chemicals in everyday consumer products and industrial processes means that several chemical compounds should be considered as emerging organic contaminants because they can be easily released into the environment [7-9]. Nowadays, the EOC group which causes the greatest concern in terms of the risk for most living communities is the group that includes pharmaceutical compounds, personal care products, veterinary drugs and other related products [3]. In addition, there are other kinds of chemical compounds that are not expected to be as dangerous as pharmaceuticals, but have an inherent risk due to the large amounts used daily.

Overall, the way in which emerging organic contaminants go from the emission source to different environmental compartments is shown in Figure 1. Most EOCs follow a similar pattern but display specific features depending on their emission point and use. For example, in the case of pharmaceutical compounds, which are one of the most studied groups of EOCs, the active pharmacological compound, either in the parent form or in the corresponding conjugate, and their metabolites are excreted by the organism. They are then flushed down into man-made sewer systems or they can reach surface waters and soils directly via run-off if they are excreted by animals, in the case of veterinary pharmaceuticals. Other classes of chemicals other than drugs, such as the compounds which are present in several daily consumer products like plasticisers or flame retardants, for example, can reach the environment via erosion of the material surface or by being leached if they are in contact with liquid substances [10]. In addition, the chemical compounds used in industry are generally flushed down into a specific sewer system connected to specific industrial sewage treatments plants.

Most of the chemical substances emitted into raw sewage may also reach the environment, as certain EOCs are only partially removed during sewage treatment. Sewage treatment plants were basically designed to remove pathogens and organic

and inorganic matter. Therefore, these conventional sewage treatments are not efficient enough to remove pharmaceuticals and other micropollutants from sewage, as demonstrated by several papers published on this topic in which certain EOCs are found in significant amounts in effluent sewage [8,11-17]. Moreover, EOCs present in effluent sewage can also reach rivers and lakes, as these environmental compartments are among the recipients of these reclaimed waters [18].

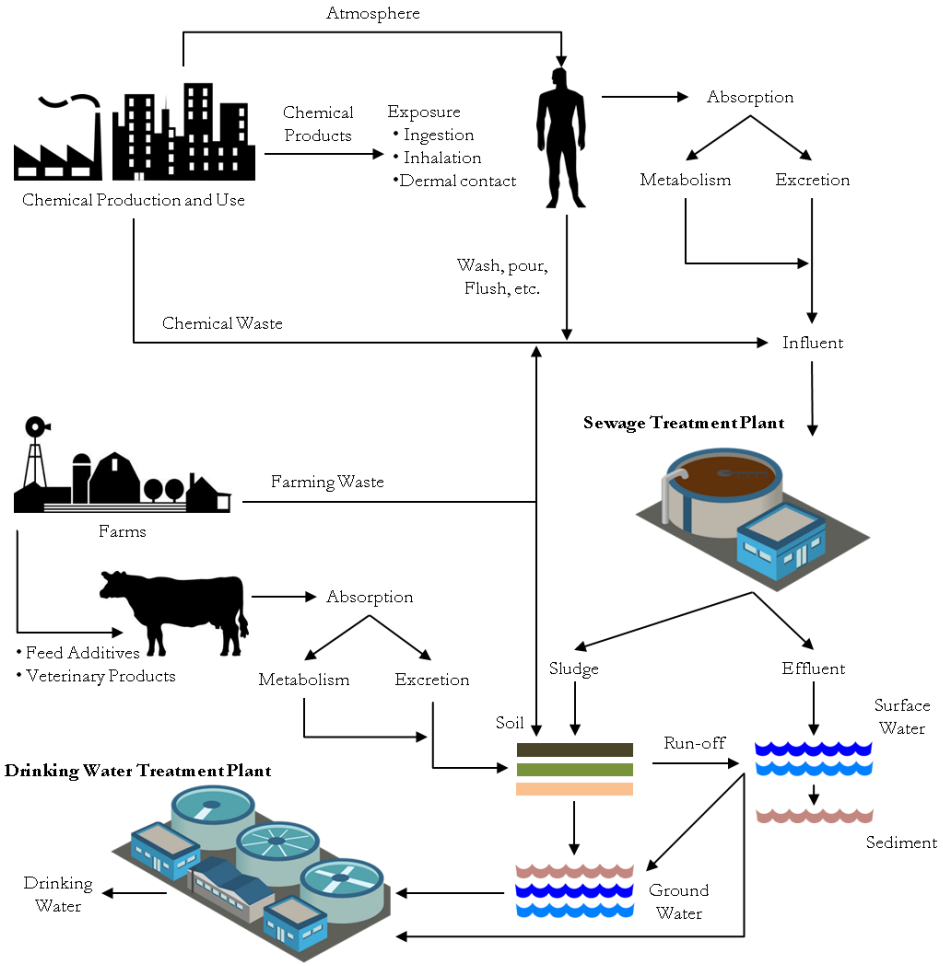

Figure 1. Fate of EOCs in the environment.

Sewage treatment was not possible until the late 19th Century when Sir Edward Frankland was able to demonstrate that filtration of sewage through porous gravel produced a nitrified effluent and showed the possibility of using an additional biological treatment. However, it was not until the early 20th Century when the development of secondary treatments became feasible through the use of activated sludge. Nowadays, activated sludge treatment is recognized worldwide as the most sustainable option for sewage treatment, as it provides effluents suitable for disposal or recycling without excessive cost. Figure 2 shows a diagram of a conventional sewage treatment plant. However, as mentioned above, these sewage treatment plants are not efficient enough to remove most of the EOCs present in sewage completely. To reduce the ecological impact of effluent sewage, some sewage treatment plants have included additional tertiary treatments in recent years to increase removal efficiency. These treatments are usually based on physical or chemical processes such as UV, chlorination, ozonation and membrane filtration, among others. Even though these treatments have proven to be more effective, some emerging organic contaminants have still been found in their respective effluents [19].

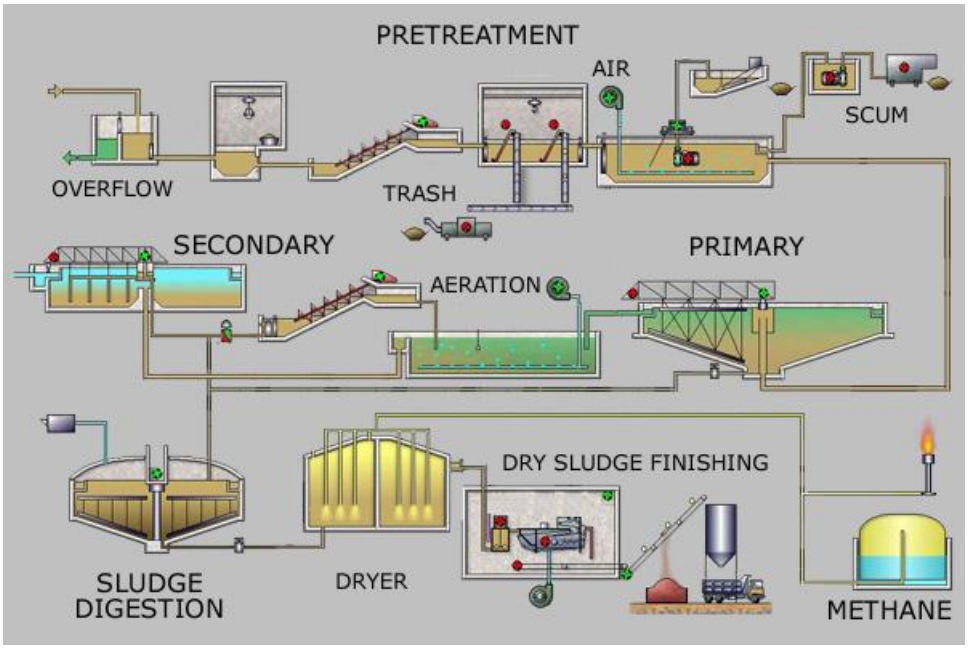

Figure 2. Process flow diagram for a conventional large-scale sewage treatment plant.

In addition to sewage effluent, sewage sludge is the resulting semi-solid waste obtained after sewage treatment, which is rich in nutrients and organic matter. However, sewage sludge is not free of chemicals (e.g. pharmaceuticals) because significant amounts of these substances are accumulated therein during sewage treatment. Therefore, their reuse on agricultural land as manure, which is the main destination, may result in some of these micropollutants reaching soils and surface or ground waters via leaching and run-off, contributing to the contamination of surrounding environment, as occurs with sewage effluents [20-25].

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1.1. Emerging organic contaminants

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As mentioned earlier, different groups of EOCs are included in this Thesis. The selected groups are glucocorticoids and polyether ionophores, which are drugs used for human and veterinary purposes, benzotriazoles, benzothiazoles and benzenesulfonamides, which are high production volume chemicals mainly used in industrial processes, and fullerenes, which are carbon-based nanoparticles that have been the focus of growing interest due to their use in several applications. In the following sections, the compounds included in each group are described in more detail, as well as their chemical properties and their ecotoxicological risk. Moreover, their occurrence in different environmental compartments is also discussed. In cases for which data is available in the literature, information about their removal during sewage treatment is reported.

1.1.1. Glucocorticoids

Corticosteroids are a class of chemical compounds that includes steroid hormones naturally produced by vertebrates in the adrenal cortex and analogues of these hormones that are synthesised in laboratories. There are two classes of steroid hormones naturally synthesised from cholesterol: the mineralocorticoids and the glucocorticoids. Their synthesis routes are shown in Figure 3. Mineralocorticoids control electrolyte and water levels in mammals and glucocorticoids are involved in a wide range of physiological processes, especially in the regulation of the metabolism of glucose. Corticosteroids have a common structure of 21 atoms of carbon distributed in four rings derived from the cholesterol molecule and, depending on their substitution, different properties are obtained. For corticosteroids, two methyl groups are bonded to C10 and C13 positions and an ethyl chain is bonded to C17. Therefore, the general structure for corticosteroids has four bounded rings (one cyclohexene (from C1 to C10), two cyclohexanes (from C5 to C10 and from C8 to C14) and one cyclopentane (from C13 to C17)), one ketone group (C3) and one alcohol group in C11.

The history of corticosteroids dates back to the Renaissance, when the suprarenal glands were discovered. Nonetheless, it was not until 1849 when Thomas Addison (1795-1860) found that a deficiency in the suprarenal hormones may cause a pathologic disease, which was known as Addison's disease. Today, it is known that this rare disease is related to a chronic endocrine disorder in which the adrenal glands do not produce sufficient steroid hormones (glucocorticoids and often mineralocorticoids). Since Addison's discovery, several diseases have been identified as being related to the abnormal function of steroid hormones, leading to a surge of

research in this field. In 1952, the complete synthesis of cortisone was accomplished and clinical research then focused on increasing the corticosteroidal effect. Corticosteroid-type drugs had been born.

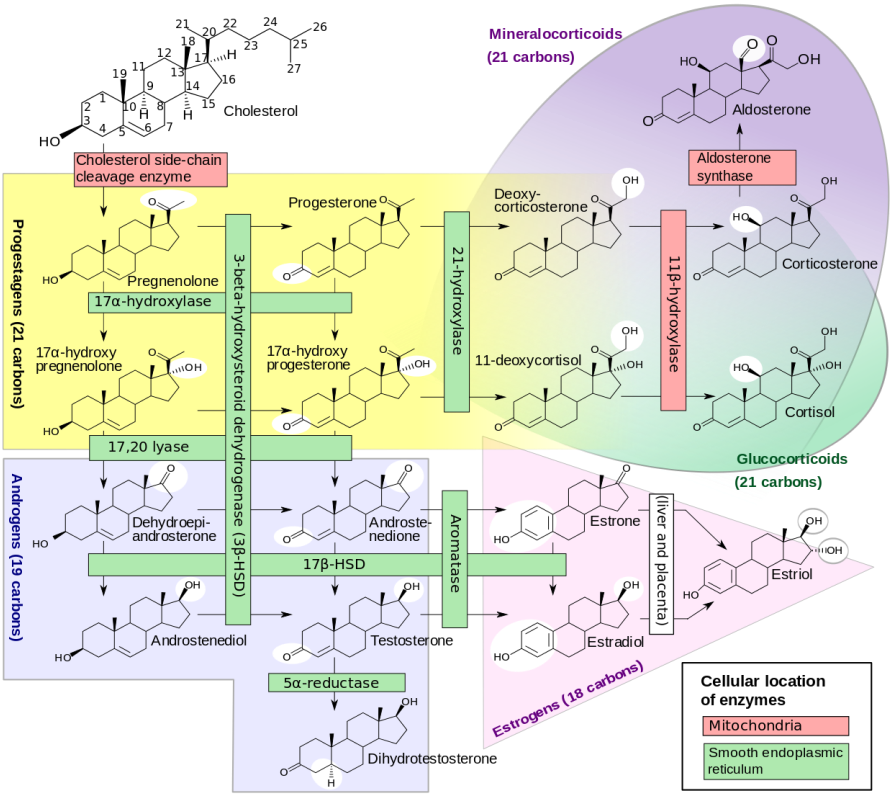


Figure 3. Synthesis of mineralocorticoids and glucocorticoids from cholesterol [26].

Nowadays, corticosteroids are one of the most commonly used anti-inflammatory drugs [27]. As mentioned above, they can be classified on the basis of their physiological effects on mineralocorticoids or glucocorticoids. However, most of them show both effects and the predominant effect is used for their classification. Due to the fact that corticoids are common hormones in mammals, they can be used both for medicine and veterinary purposes. Modifications to the 21-carbon steroid skeleton selectively alter the degree of anti-inflammatory activity, the duration of activity, the protein-binding affinity and the metabolic consequences.

The effects of these modifications are linked to the skeleton ring into which the specific group is added. In the first ring, the double bond between C4 and C5 and the ketone group in C3 is necessary for adrenocorticosteroidal activity and the introduction of an additional double bond between C1 and C2 enhances glucocorticoid and anti-inflammatory activity without affecting mineralocorticoid activity, which results in a higher glucocorticoid/mineralocorticoid ratio (e.g. prednisone and prednisolone drugs). In addition, halogenation, mainly with fluorine or chlorine, of C6 or C9 carbons in the second ring enhances both glucocorticoid and mineralocorticoid activity but not in the same ratio. The addition of an oxygen molecule, such as an alcohol or ketone group, in the third ring in C11 increases the anti-inflammatory potency group. Moreover, methylation or hydroxylation in C16 in the fourth ring suppresses the retention of sodium and so no mineralocorticoid effect is observed (e.g. betamethasone or dexamethasone). Finally, the esterification of the alcohol at C21 or the addition of an acetonide group between C16 and C17 allows more lipophilic drugs to be obtained (e.g. triamcinolone acetonide). All of the anti-inflammatory steroids currently used in veterinary medicine have an α-hydroxyl group in C17. Table 1 lists the most widely used corticosteroid drugs, their chemical formula, glucocorticoid and mineralocorticoid activity, and half-life duration of the effect.

Table 1. Chemical formula and relative potencies of commonly used corticosteroids [28,29].

Name	**Chemical formula**	**Relative glucocorticoid activity**	**Relative mineralocorticoid activity**	**Duration of effect ($t_{1/2}$ in hours)**
Cortisol	$C_{21}H_{30}O_5$	1	1	8
Cortisone	$C_{21}H_{28}O_5$	0.8	0.8	8-18
Prednisone	$C_{21}H_{26}O_5$	5	0.8	16-36
Prednisolone	$C_{21}H_{28}O_5$	5	0.8	1-36
Methylprednisolone	$C_{22}H_{30}O_5$	5	0.5	18-40
Dexamethasone	$C_{22}H_{29}FO_5$	25	0	36-54
Betamethasone	$C_{22}H_{29}FO_5$	25	0	36-54
Triamcinolone	$C_{21}H_{27}FO_6$	5	0	12-36
Triamcinolone acetonide	$C_{24}H_{31}FO_6$	30	0	12-36
Fludrocortisone	$C_{21}H_{29}FO_5$	10	125	12-36
Fludrocortisone acetate	$C_{23}H_{31}FO_6$	15	200	24
Isoflupredone	$C_{21}H_{27}FO_5$	25	25	48-72
Flumethasone	$C_{22}H_{28}F_2O_5$	120	0	48-72
Deoxycorticosterone acetate	$C_{23}H_{32}O_4$	0	20	-
Aldosterone	$C_{21}H_{28}O_5$	0.3	200-1000	-

Synthetic corticosteroid drugs are used to treat a variety of diseases due to their anti-inflammatory properties. Thus, they are used in rheumatology, gastroenterology, oncology, neurology, dermatology and ophthalmology, as well as to treat allergies and induce labour [30]. However, some side effects are also related to the use of this kind of drugs, which reduce their long-term prescription to patients. Some of these side effects may be severe, such as psychosis, anxiety or depression, hypertension, metabolic and endocrine dysfunctions, which may cause hyperglycaemia or erectile dysfunction, osteoporosis, ulcer and myopia. Moreover, corticosteroids have a low but significant teratogenic effect, especially in pregnant women or mammals. One of the most important side effects observed in corticoid drugs is that they affect water retention and the carbohydrate, protein and lipid metabolism of vertebrates. A long-term sub-therapeutic dosage of this kind of drug results in weight increment and high muscle/fat ratio due to the retention of water in the muscle. This fact was used in the past for growth promotion in cattle but the use of corticoids as growth-promoting agents in livestock farms was banned in the European Union (EU) in 1990 [31]. Thus, the use of veterinary drugs, especially hormones, to increase growth efficiency started after the Second World War, when the recognition of the growth-promoting properties of oestrogenic steroids hormones (oestrogens) led to their introduction into animal feed to increase meat production, which was necessary due to difficulties in obtaining food for the population. As an example, as a cheap and better absorbed analogue of the natural hormone oestradiol-17β, diethylstilbestrol (DES) became the preferred growth promoter for cattle, sheep and poultry in many countries. In the 1970s, concerns about its safety were raised when DES was confirmed to be a human carcinogen, but the health risk was considered insignificant by the scientific community because DES residues in meat were very low compared to the amount used as a drug. Therefore, the use of DES as a growth promoter continued in some Member States of the EU longer than in the United States of America (USA) but it was finally banned in 1987 in the EU and in 1972 in the USA [32]. However, the same practices (fraudulent or not) continue today with new drugs which are not yet banned.

The case of corticoids is a delicate issue because of the great potency of these drugs for tackling a multitude of diseases. As already mentioned, corticoid dosage for growth promotion is currently banned in the EU. However, they are used for therapeutic purposes and, therefore, they are included in the corresponding European regulation for maximum veterinary residues allowed in foodstuffs (EU Nº 37/2010) [31]. As an example of the use of glucocorticoids for both human and veterinary medicine, the total amounts used in the UK in 2006 were around 4,700

Kg, with veterinary use accounting for approximately 7% of this value [27]. The extended use of glucocorticoid drugs both in human and veterinary medicine can transfer these chemicals to the environment in different ways [7,8,11,33].

As mentioned above, one of the main ways that pharmaceuticals are released is excretion by the organism of non-metabolised/adsorbed drugs, either in the parent form or in the corresponding conjugate form. For example, around 50% to 90% of glucocorticoids are quickly excreted in urine and faeces. The excreted substances are then emitted into raw sewage and they may later reach the environment because they escape degradation in sewage treatment plants. In addition, waste containing pharmaceuticals and the disposal of unused or expired drugs by manufacturers may contribute to environmental contamination, but the former intrusion route is the most widely accepted. In the case of veterinary drugs, additional intrusion routes should be considered besides those mentioned above. The drug dosage to animals is often combined with fodder, or directly into the water in fish farms, and these chemicals can therefore easily reach soil and groundwater, as no previous sewage treatment is performed. [34]. Moreover, veterinary residues present in urine or faeces and feed from cattle can also reach ground and surface waters by run-off due to rainfalls, thereby contributing to their contamination.

Glucocorticoids are considered to be endocrine-disrupting chemicals (EDCs), which are a category of environmental pollutants that, at certain doses, can interfere with the endocrine system in mammals and produce adverse developmental, reproductive, neurological and immune effects in both humans and wildlife. Hormones, pharmaceuticals, dioxins, polychlorinated biphenyls and plasticisers are a few examples of EDCs [35]. Therefore, these substances present a risk to the exposed organisms in terms of impairing immune function, reproduction or development, and the ecotoxicological risk of glucocorticoids is apparent, as they are relatively soluble in water and their log P values range from 1 to 4 [27]. Nonetheless, the amount of these pollutants actually introduced in the environment is small, but a continuous drip over time into surface waters may result in a relatively high concentration or long-term exposure for aquatic organisms.

In view of this exposure, some researchers have focused their interest on the presence of these compounds in the environment. Table 2 shows the results obtained in recent years of the occurrence of certain glucocorticoids in environmental waters in different countries. Most of the studies focus on sewage samples from European countries and China. The highest concentrations of

glucocorticoids were found in wastewater from a hospital in the Netherlands, with prednisolone being the most abundant, at concentrations around 2,000 ng/L [36].

Table 2. Occurrence of the most relevant glucocorticoids in environmental waters.

Compound	Matrix	Conc. (ng/L)	Country	References
Betamethasone/ Dexamethasone	Industry WW	90	The Netherlands	[36]
	River water	<0.01-8.0	China	[37,38]
	River water	<0.01-0.06	Hungary	[39]
	STP effluent	n.d-0.09	China	[37,38,40,41]
	STP effluent	7	France	[42]
	STP influent	0.3-22.6	China	[37,40,41]
	STP influent	15	France	[42]
	STP influent	9.4	Japan	[43]
Cortisol (Hydrocortisone)	Hospital WW	275-301	The Netherlands	[36]
	Industry WW	13	The Netherlands	[36]
	River water	<0.01-20	China	[37,38]
	River water	<0.17-2.67	Hungary	[39]
	STP effluent	n.d.-1.9	China	[37,38,40,41]
	STP effluent	63	France	[42]
	STP influent	9.2-120	China	[37,40,41]
	STP influent	53	France	[42]
Cortisone	Hospital WW	381-472	The Netherlands	[36]
	Industry WW	26	The Netherlands	[36]
	River water	<0.02-28	China	[37,38,40]
	STP effluent	n.d.-0.88	China	[37,38,40,41]
	STP effluent	n.d.-229	France	[42,44]
	STP influent	4.6-86	China	[37,40,41]
	STP influent	174	France	[42]
Flumethasone	River water	70.7-85.1	Spain	[45]
Methylprednisolone	River water	<0.01-0.41	China	[37,38]
	STP effluent	n.d.-0.03	China	[37,38,41]
	STP influent	<0.08-2.0	China	[37,41]

Table 2. (Cont.).

Compound	Matrix	Conc. (ng/L)	Country	References
Prednisolone	Hospital WW	315-1,918	The Netherlands	[36]
	Industry WW	247	The Netherlands	[36]
	River water	<0.01-1.8	China	[37,38]
	River water	<0.04-0.58	Hungary	[39]
	STP effluent	n.d.-0.72	China	[37,38,41,43]
	STP influent	1.5-7.5	China	[37,41]
	STP influent	17	Japan	[43]
Prednisone	Hospital WW	117-545	The Netherlands	[36]
	River water	n.d.-2.4	China	[37,38,40]
	STP effluent	n.d.-0.18	China	[37,38,40,41]
	STP effluent	n.d.-10.0	France	[44]
	STP influent	n.d.-8.5	China	[37,40,41]
Triamcinolone acetonide	Hospital WW	14-41	The Netherlands	[36]
	River water	<0.63	Hungary	[39]
	STP effluent	3	France	[42]
	STP effluent	14	The Netherlands	[36]
	STP influent	40	France	[42]

WW: waste water.

Also in the same study, industrial wastewaters from a factory producing veterinary medicines were analysed and some of these compounds (mainly dexamethasone and prednisolone) were found at relatively high concentrations, compared with raw influents in urban STPs. As can be seen in Table 2, the frequency of detection and average concentrations determined were higher in influent sewage samples than effluents sewage samples. Their concentration ranges were from a few ng/L to hundreds ng/L, with the endogenous glucocorticoids (cortisol and cortisone) being the compounds present at higher concentrations, which are also used as pharmaceuticals. Therefore, the intrusion of these compounds into STPs is a reality and, as can be seen in the data from Table 2, several glucocorticoids are still present in STP effluent sewage. The concentrations of these compounds in effluent sewage ranged from sub ng/L to a few ng/L for synthetic glucocorticoids such as betamethasone, prednisone or triamcinolone acetonide, and from sub ng/L levels to hundreds ng/L for endogenous compounds, cortisol and cortisone, which are naturally synthesised and excreted by humans and

wildlife. Moreover, the concentrations of these compounds reported by the studies conducted in European countries, mainly in France, are higher than those reported in the studies carried out in China. However, their occurrence in effluent sewage samples from STPs located in the Netherlands are much lower than the others and only one compound (triamcinolone acetonide) was determined, since other glucocorticoids were not detected. Although most of them were determined in effluent sewage, their concentrations were relatively lower compared to other pharmaceutical compounds, which were found at μg/L levels.

With respect to the removal of glucocorticoids during different sewage treatments, a number of authors have presented studies on this issue. For example, Kitaichi *et al.* [43] analysed samples from different sampling points during sewage treatment in an STP located in Japan and subjected to an additional chlorination treatment. They include fluocinolone acetonide, prednisolone acetate, hydrocortisone acetate, betamethasone valerate and betamethasone dipropionate, as well as some of the most common glucocorticoids shown in Table 2. Only prednisolone (17 ng/L), betamethasone and dexamethasone (9.4 ng/L), hydrocortisone acetate (3.8 ng/L) and betamethasone valerate (8.6 ng/L) were determined in the influent sewage samples. After primary treatment, their concentrations remained equal and, after secondary and chlorination treatments, only betamethasone valerate was still found (1.3 ng/L) in effluent sewage. Based on this study, it seems that the major removal of glucocorticoids in sewage treatments is achieved during the secondary treatment, while chlorination appears not to influence their removal. However, only one compound is detected after the secondary treatment and the results presented may not be the same for other glucocorticoids. Overall, the removal efficiency of glucocorticoids in urban STPs is estimated to be higher than 96% [37] and, therefore, low concentrations of these compounds are expected in surface waters.

As regards tertiary sewage treatments, they have not yet been studied thoroughly in terms of the removal of glucocorticoids and only a couple of studies using them have been conducted in lab-scale experiments. The first of these studies investigated the use of membrane bioreactors which were tested in lab-scale experiments for the removal of corticoids, obtaining removal efficiencies higher than 93% [46]. The other study focused on the electrocoagulation process, which is more efficient than chemical coagulation and presents various advantages, such as low cost, easy handling and high efficiency in terms of the removal of organic matter [47]. However, the sludge ratio obtained is much higher than in other treatments. The

removal of pharmaceuticals is probably related to their attachment to colloidal particles generated during electrochemical reactions. Hence, Arsand *et al.* [47] applied this treatment in lab-scale experiments for the removal of dexamethasone in hospital wastewaters and only ~38% of dexamethasone was removed by this process.

Data about the occurrence of these compounds in surface waters is limited compared with their presence in sewage. However, several glucocorticoids were found at very low concentrations, normally at sub ng/L levels, in river waters from China, as shown in Table 2. As discussed previously, these low concentrations are expected because concentrations reported in STP effluents are low. The concentrations reported in these studies may suggest that additional intrusion routes of these compounds into surface waters are not obvious and so the main emission source is probably STP effluents. For example, Gros *et al.* [48] analysed different kinds of surface waters in a multiresidue study, which included glucocorticoid betamethasone. In this study, river water, sea water, drinking water and reservoir water from different areas of Catalonia (Spain) were analysed but betamethasone was not found in any of them. A few additional papers report the occurrence of less commonly used glucocorticoids in sewage (data not included in Table 2). Piram *et al.* [42] determined budesonide, flunisolide, fluocinolone acetonide and triamcinolone, both in influent and effluent sewage from an STP located in France at concentrations between 0.3 and 31 ng/L in influent sewage and between 3 and 30 ng/L in effluent sewage. The authors suggest that the highest concentrations found for some compounds in effluent sewage may be due to the hydrolysis of glucocorticoid conjugates during the STP treatment.

In addition to studies focused on the determination of target glucocorticoids and their occurrence in environmental waters, there are also a few papers that focus on the measurement of glucocorticoid activity in water using bioassay tests, which can be applied for monitoring the quality of the aquatic environment for a specific set of compounds. Studies on water sources in the USA [49] and the Netherlands [50,51] showed glucocorticoidal activity in most of the water sources included in these studies. Nonetheless, the current data and present knowledge of the environmental implications of these compounds is insufficient, but this prevalent contamination may negatively affect wildlife and the human population in the future.

Environmental pollution from pharmaceuticals, and particularly glucocorticoids, is not limited to environmental waters. As mentioned earlier, the resultant sewage

sludge obtained during sewage treatment in STPs is a matrix potentially polluted by pharmaceuticals and it may be one of the intrusion routes by which pharmaceuticals are released into other environmental matrices like soils and sediments. However, the information about the occurrence of glucocorticoids in environmental solid matrices is very limited. Fan *et al.* [41] and Liu *et al.* [40] analysed different sludge samples from STPs located in China. The former found some glucocorticoid residues, mainly cortisone and prednisone, ranging from 0.05 to 0.37 ng/g in different sludge samples obtained during sewage treatment, such as anaerobic sludge, anoxic sludge, aerobic sludge or dehydrated sludge. In contrast, the latter did not find glucocorticoid residues in similar kinds of samples. The studies regarding the occurrence of glucocorticoids in soils and sediments are even more limited. Only one paper focuses on the analysis of these kinds of samples in a multiresidue study of human and veterinary drugs [52]. However, only the glucocorticoid prednisolone was included in that study, being detected in one river sediment sample from Spain below the limit of quantification (LOQ).

As shown above, the existing literature clearly confirms the presence of glucocorticoids in environmental waters, especially in sewage. Moreover, conventional sewage treatments have been shown to be quite effective for their removal, but not completely so, as some glucocorticoids are still found in effluent sewage samples. Their occurrence in sewage sludge and other environmental solid matrices needs further attention in the future, since very few papers regarding this issue have been published to date. However, as sewage sludge is probably not the main destination of this kind of environmental pollutants, more studies are still necessary to corroborate this hypothesis.

1.1.2. Polyether ionophores

Nowadays, more than one hundred polyether ionophores are known and their major commercial use is to control the disease coccidiosis [53-55]. Polyether ionophores are a very large group of naturally occurring compounds generated by bacterial fermentation processes (e.g. *Streptomyces spp.*). Coccidiosis is a common protozoan infection of the intestinal tract of farm animals which predominantly affects the young. The disease spreads through contact with infected faeces or ingestion of infected tissue. Diarrhoea is the primary symptom but, in most animals, the disease is asymptomatic, while they may suffer severe symptoms and death. Normally, the bacteria causing coccidiosis are species-specific, which means that there are certain bacteria for each particular animal species. However, there are

exceptions, such as toxoplasmosis. Since polyether ionophores exhibit a broad range of biological, antibacterial, anticoccidial, antiviral, antiparasitic and insecticidal activity, they are extensively used on livestock farms, mainly for cattle, pigs and chickens. Therefore, it is not rare for ionophore-type coccidiostats, which include polyether ionophores, to be one of the main groups used in veterinary medicine [56-61], after tetracyclines. An example of the usage of antibiotics, including polyether ionophores, in tonnes of active ingredient sold in the United Kingdom in 2010 is shown in Figure 3.

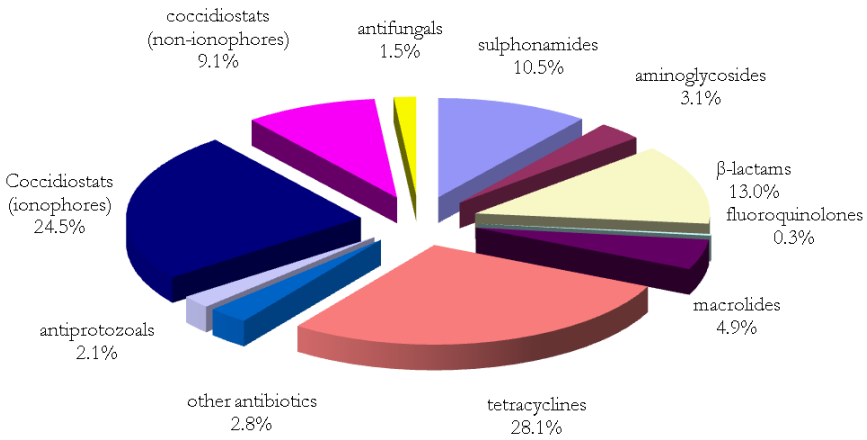
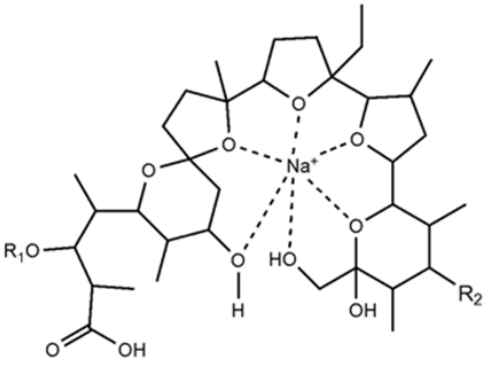


Figure 3. Sales of antibiotic products (tonnes active ingredient) in the UK in 2010 [61].

Polyether ionophores are large (molecular weight over 500 Da) and hydrophobic molecules characterised by having multiple tetrahydrofuran and tetrahydropyran rings connected between them by aliphatic chains, as can be seen in Figure 4. In addition, they have a free carboxylic acid group at one end and a terminal alcohol group at the other, as well as other functional oxygen groups, mainly alcohols, along their structure. All polyether ionophores have the ability to form a pseudo-macrocyclic ring by hydrogen bonding between the carboxylic acid and the terminal alcohol group. This spatial conformation concentrates all of the oxygen groups at the centre of their structure and, therefore, they can complexate mono or divalent cations using ether groups as chelating agents. In addition, polyether ionophores are highly selective for specific cations due to their inner space when the molecule adopts the cyclic ring formation [62]. As an example, Figure 4 shows the spatial conformation of the polyether ionophore monensin complexing a sodium cation. The neutral complexes with cations are formed more preferably because the

carboxylic group is deprotonated at physiological pH. However, recent studies show that polyether ionophores can also act as neutral molecules and wrap the metal cations, which results in a charged complex. These metal ionophore molecules (either neutral or charged) are also lipophilic and, therefore, they are able to transport ions across the lipid bilayer of the cell membrane. Thus, ionophores disrupt the Na^+/K^+ gradient and increase the osmotic pressure inside the cell causing death, because coccidia cells do not have osmoregulatory organelles. Due to their natural production using certain bacteria, several analogues of each polyether ionophore are obtained together with the parent compounds. For example, monensin has analogues known as monensin A (the parent compound), B, C and D. They differ in terms of one methylene and/or hydroxyl group substitution in different positions of the molecule.



Monensin	A	$R_1=CH_3$,	$R_2=H$
	B	$R_1=H$,	$R_2=H$
	C	$R_1=CH_3$,	$R_2=OH$
	D	$R_1=H$,	$R_2=OH$

Figure 4. Pseudo-macrocyclic structure of monensin and their analogues.

As mentioned above, the use of polyether ionophores is limited to veterinary medicine, primarily for the treatment of coccidiosis on poultry and livestock farms, but they are also used as growth promoters in ruminants. They are a common ingredient in most antibiotic drugs used in veterinary medicine, such as sulphonamides, and they are related to the interaction of these drugs with the intestinal microbial population. However, recent studies have shown that several polyether ionophores exhibit anticancer activities (mainly salinomycin) in humans and so they are currently widely believed to be candidates to be tested as anticancer drugs. With regard to the adverse effects of polyether ionophores, they are considered to be free from side effects and toxicity if they are used in the correct way and for the appropriate species. However, it has been observed that they can cause myocardial insufficiency and muscle weakness, which is also the case in humans observed who have accidentally ingested monensin [54,63].

The use of antibiotics for growth promotion started in the 1940s, when it was observed that animals fed with dried mycelia of *Streptomyces aureofacies* containing chlorotetracycline increased their weight. Polyether ionophores were recognised as a separate class of antibiotics in 1967, when the structure of monensin was discovered. From the 1950s onwards, each European country approved its own regulations with respect to the use of antibiotics in animal feed and it was not until 1970 that the first European regulation (Council Directive Nº 70/520/EEC [64]) concerning this issue was approved. In 2003, a new regulation (Council Regulation Nº 1831/2003/EC [65]) replaced the earlier directive, banning the use of antibiotics other than coccidiostats and histomonostats, stating that a veterinary prescription is necessary for their use from 2006 onwards (for coccidiostats in 2013) [66]. The polyether ionophore antibiotics approved in the EU to prevent coccidiosis are listed in Table 3.

Table 3. Name, chemical formula and target animal of the authorised coccidiostats in EU as feed additives.

Name	Chemical formula	Animals
Monensin	$C_{34}H_{54}O_8$	Chickens and turkeys
Lasalocid acid	$C_{36}H_{62}O_{11}$	Chickens and turkeys
Salinomycin	$C_{42}H_{70}O_{11}$	Chickens and rabbits
Narasin	$C_{43}H_{72}O_{11}$	Chickens
Maduramicin	$C_{47}H_{80}O_{17}$	Chickens and turkeys
Semduramicin	$C_{45}H_{77}O_{16}$	Chickens

Due to the extensive use of polyether ionophores as veterinary feed additives, their fate is expected to be in the environment, as is the case with other veterinary medicines, such as macrolides, sulphonamides, tetracyclines and quinolones. However, coccidiostats, such as polyether ionophores, have not received the same attention and only a few published studies are available regarding the occurrence of these compounds in environmental matrices. Nonetheless, concern is growing about the amount of veterinary medicines in the environment, because there is a threat of a daunting public health risk due to the emergence of resistant bacteria [12,14,67,68]. As a result, they are considered to be emerging pollutants [69] and risk assessment studies have shown that polyether ionophores might be an environmental risk to living organisms in water, soils and sediments.

As explained in the previous section, the intrusion routes of veterinary medicines into the environment are not dissimilar to those shown for pharmaceutical

compounds. However, it is necessary to consider specific methods for these environmental contaminants. The main routes are the excretion of non-metabolised medicines by animals and the administration of medicines combined with fodder. Moreover, the substances excreted in faeces can be transferred to soils if they are used as manure. Therefore, veterinary medicines can easily reach soils and ground waters by leaching or may reach surface waters via run-off. As an example, around 80% of lasalocid acid administered to chickens is excreted in urine and faeces. While the presence of veterinary medicines in sewage is not expected, a number of papers have already reported their presence [12,14,67]. Some of these papers suggest that discharges from veterinary clinics and industries, run-off from agricultural applications, household use for pets and wastes from meat processing plants and abattoirs may be complementary intrusion routes of veterinary medicines into the municipal sewer systems.

Most of the papers regarding the occurrence of polyether ionophores into the environment focus on surface waters, soils and sediments, as shown in Table 4. The concentration ranges of these compounds are strongly dependent on the area involved. Kim *et al.* [70] also suggested that seasonal variability may affect their concentrations. Usually, these compounds are found at levels of a few ng/L in surface waters, mainly river water samples. Most of the studies were conducted in the USA, where polyether ionophores were the second top-selling veterinary medicine group in 2009 and 2010, as reported by the US Food and Drug Administration (FDA) [71,72]. The most studied compounds were monensin and salinomycin because they are the most widely used. However, there are certain values which stand out from the rest, such as those reported in the studies by Thompson *et al.* [73], Watkinson *et al.* [74] and Kurwadkar *et al.* [75]. The first two found hundreds of ng/L for monensin and salinomycin in surface waters, which are influenced by surrounding agricultural areas, which may therefore result in these higher concentrations. Kurwadkar *et al.* [75] found more than 5,000 ng/L of monensin in river water but their results may be strongly affected by the analytical method used (enzyme-linked immunosorbent assay (ELISA)), because this is a very high concentration for this compound in surface water. For example, a similar concentration was only found in the case of run-off waters from soils that were fertilised with litter and, therefore, it is more probable that these waters contain a high amount of polyether ionophores. In addition, the presence of these compounds in Spanish rivers was studied by Iglesias *et al.* [45] and Martínez-Villalba *et al.* [62]. The former found monensin, maduramicin and salinomycin in river water

samples from Galicia, while the latter did not find polyether ionophores in the samples analysed from Catalonia.

The occurrence of polyether ionophores in sewage samples has not received the same attention as other aqueous matrices, probably due to the fact that they are not expected to be found in this kind of samples, as discussed earlier. However, there are two studies regarding the analysis of sewage samples in China [76] and Australia [74]. While in China no residues of polyether ionophores were found, research into samples from an Australian STP found quite high concentrations of salinomycin in influent sewage and monensin in effluent sewage. Zhou *et al.* [76] also analysed sewage sludge samples from the STP located in China, but no polyether ionophore compounds were found. Therefore, more studies are necessary to expand the current information about the fate of these compounds in sewage sludge, because there is no information available to date.

Table 4. Occurrence of polyether ionophores in the environment.

Compound	Matrix	Conc. (ng/L, ng/g)	Country	References
Lasalocid acid	River water	10.7-27.7	Canada	[77]
Maduramicin	River water	23	Spain	[45]
Monensin	Agricultural watersheds	2-843	Canada	[73]
	River water	2-150	Australia	[74]
	River water	2.4-22.1	Canada	[77]
	River water	14.6-16.7	Spain	[45]
	River water	n.d.-5130	USA	[70,75,78,79]
	Run-off from agricultural lands	2-37	USA	[80]
	Run-off from litter-fertilised lands	n.d.-2389	USA	[81]
	STP effluent	n.d.-20	Australia	[74]
	Sediment	n.d.-31.5	USA	[70,79]
	Soil	0.4	Denmark	[82]
	Soil from litter-fertilised lands	5-183	USA	[81]

Table 4. (Cont.).

Compound	Matrix	Conc. (ng/L, ng/g)	Country	References
Narasin	Agricultural watersheds	3-19	Canada	[73]
	River water	n.d.-60	USA	[70,78,79]
	Run-off from litter-fertilised lands	n.d.-348	USA	[81]
	Sediment	0.7	Denmark	[82]
	Sediment	n.d.-30.1	USA	[70,79]
	Soil	2.2	Denmark	[82]
Salinomycin	Agricultural watersheds	2-13	Canada	[73]
	River water	n.d.-150	Australia	[74]
	River water	15.6-17.4	Spain	[45]
	River water	n.d.-40	USA	[70,78,79]
	Run-off from litter-fertilised lands	n.d.-9022	USA	[81]
	STP influent	n.d.-300	Australia	[74]
	Sediment	0.4	Denmark	[82]
	Sediment	n.d.-16.3	USA	[70,79]

In addition, there are a few papers reporting the occurrence of monensin, salinomycin and narasin in soil and sediments from the USA [81] and Denmark [82]. As in the case of surface waters, their concentrations are highly influenced by the sampling site, as observed by the difference in concentrations for monensin, for instance, which were found to be 0.4 ng/g in agricultural soil from Denmark and at 2,389 ng/g in a litter-fertilised soil from the USA. Schlüsener *et al.* [83] analysed soil samples fertilised with liquid manure which contained salinomycin, but no residues of this compound were found, probably due to its degradation as reported in another paper by the same authors [25]. The degradation of polyether ionophores in soils is also supported by others papers in which the sorption and degradation kinetics for certain polyether ionophores were studied [84-88]. In addition, the removal of polyether ionophores was investigated in constructed wetlands [89], obtaining removal efficiencies around 30% for monensin, salinomycin and narasin.

With the current data presented above, it has been shown that polyether ionophore antibiotics are often present in several environmental matrices at levels of a few ng/L in the case of waters and a few ng/g in soils and sediments. Surface water is the most common destination for these compounds as a result of the use of contaminated litter and manure to fertilise agricultural soils, before leaching and run-off of these compounds lead them to reach surface waters. These compounds have been also determined in sewage but the information about their behaviour through different sewage treatments and their occurrence in sewage samples is very limited and more investigation into this kind of samples is necessary to assess the presence of these compounds in urban STPs.

1.1.3. Benzotriazoles, benzothiazoles and benzenesulfonamides

High production volume (HPV) chemicals are chemical compounds produced or imported in quantities higher than 1,000 tonnes per year in at least one European member country or in the European Union, as defined by the Organisation for Economic Co-operation and Development (OECD) [90], and quantities of 1 million pounds or more per year in the United States [91]. Since very large amounts of several chemicals are used today, high production is a proxy for high exposure, with inherent ecotoxicological risks associated for health and the environment.

The origin of existing chemical assessments started in a Council Decision in 1987, which established a number of programmes to investigate existing chemicals systematically. In 1991, the member countries focused their research on high production volume chemicals based on the assumption that production volume is a substitute for data on occupational, consumer and environmental exposure. In 1998, an international programme was initiated which aimed to assess the potential hazard of high production volume chemicals. The HPV Programme was supported by the International Council of Chemical Associations (ICCA) and was carried out cooperatively by the chemical industry, with more than 4,000 chemical compounds being included.

The aforementioned list of compounds included benzotriazole, benzothiazole and benzenesulfonamide derivates, which are extensively used for several household and industrial applications. For example, between 2000 and 2010, the annual production of benzotriazoles was about 9,000 tonnes per year worldwide [92], while the production of benzothiazole derivates used in the rubber industry was about 38,000 tonnes per year just in Western Europe in the 1980s [93]. The annual

production of *para*-toluenesulfonamide was between 500-5,000 tonnes in United States in 1998 [94]. However, current data on the chemical production of these compounds is scarce.

Benzotriazoles are heterocyclic substances containing the 1,2,3-benzotriazole skeleton, with their basic structure being two fused rings with three nitrogen atoms. Due to the aromatic features of these compounds, tautomeric species can exist in its five-member ring, as shown in Figure 5. The isomer A is the predominant structure at ambient temperature, but it has been observed that the proton migrates rapidly between positions 1 and 3. This feature confers a certain acidic property ($pK_a>8$) to benzotriazole compounds and they can even act as a Brønsted base by accepting a proton in the lone pair electrons located on nitrogen atoms ($pK_a<0$). Despite having acid-base properties, the lone pair electrons can also bind to metal compounds, mainly copper, forming stable coordination compounds which confer the corrosion inhibitor properties to benzotriazoles. However, the exact structure of copper-benzotriazole complexes is still unclear. Because of this anticorrosion property, benzotriazoles are extensively used as corrosion inhibitors in several fluids which are used in contact with metals, such as aircraft de-icing and anti-icing fluids or washing powders and tablets used every day in our homes as detergents [95]. The most commonly used benzotriazole compounds are 1-H-benzotriazole and tolyltriazole, mixture which is a technical mixture of 4- and 5-methyl-1H-benzotriazole. Moreover, some phenolic benzotriazole derivates are widely used in plastics and other polymeric materials as UV filters [96].

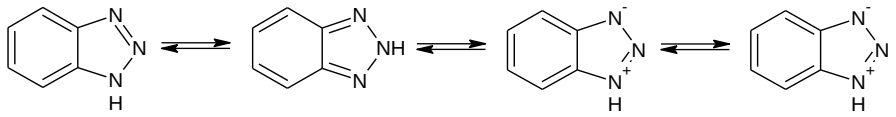


Figure 5. Tautomeric structures of 1-H-benzotriazole.

Benzothiazoles are also aromatic heterocyclic compounds consisting of a 1,3-thiazole ring fused to a benzene ring. All of their derivates are usually made by substituting the desired chemical group in the carbon atom placed in position 2 (between the N and S atoms), while the nine atoms of the bicycle and the attached substituent are coplanar. In contrast to benzotriazoles, acidic or basic features are not expected from benzotriazole compounds, if the attached substituent does not

have ionisable properties *per se*, e.g. 2-aminobenzothiazole (pK_a=7.8). The 2-substituted benzothiazoles are thermally stable compounds with numerous applications in dyes such as thioflavin and, moreover, some drugs contain a benzothiazole group. As mentioned above, one of the major applications of benzothiazoles is in the rubber industry, in which they are used as vulcanisation accelerators, with 2-mercaptobenzothiazole being extensively used [97]. While several benzothiazole derivates are used in a range of applications, their parent compound is not really widely used for industrial applications. For the sake of interest, a natural derivate of benzothiazole (luciferin) can be found in fireflies, acting as the light-emitting component in these insects.

Benzenesulfonamides are aromatic compounds based on one benzene ring with a sulfonamide group substituent as a basic structure. Moreover, different derivates can be obtained by the addition of methyl in the benzene ring, obtaining toluenesulfonamides, or by the *N*- substitution in the sulfonamide group. Because of the existence of the sulfonamide group in these compounds, they can act as acids (pK_a>10), being the proton donor of the amide group. Benzenesulfonamides are used in several applications and they are generated as undesired products in some production processes or as degradation product from other compounds. For example, benzenesulfonamide is used as an intermediate reactive for the synthesis of dyes and disinfectants, while toluenesulfonamide is used in enamels and fingernail polishes and as a plasticiser [98,99]. Moreover, toluenesulfonamide is also obtained during saccharin production. However, one of the major sources of toulenesulfonamide is found in the degradation of Chloramine-T (*N*-sodium-*N*-chloro-*para*-toluenesulfonamide) which is an antimicrobial agent, and *para*-toluenesulfonamide has been used as a marker residue for this disinfectant [94].

As stated in the objectives of the HPV chemicals programme, ecotoxicological risks for these compounds are expected because of the large amounts used. Most benzotriazole, benzothiazole and benzenesulfonamide derivates have low toxicity to humans but some of them show toxic or hazard effects in other organisms, and 1-H-benzotriazole is a suspected human carcinogen, as reported by the Dutch expert committee on occupational standards [100]. For example, two benzotriazole derivates have been shown to be mutagenic in bacterial systems, while benzotriazole has a toxic effect on plants [101,102]. Moreover, it has been shown that 1-amino-benzotriazole is a potent mechanism-based inhibitor of cytochrome P-450s, which is involved in both detoxifying many xenobiotic substances and activating compounds into carcinogens in mammals. Benzothiazole derivates mainly show

antiviral activity, especially the 2-mercaptobenzothiazole compound, and they also act on bacteria, yeast and fungi. In addition, it has been observed that benzothiazole decreases activated sludge respiration by inhibiting ammonia oxidation. The toxicity of benzothiazole compounds is probably due to its metal-chelating properties and a result of the interaction with certain groups present in proteins. With respect to benzothiazole metabolites, potential mutagenic and carcinogenic metabolites, such as aromatic hydroxylamines, are generated by the benzothiazole ring-opening reaction [103]. The toxicity of benzenesulfonamides is classified as moderate since *para*-toluenesulfonamide is slightly toxic to algae but non-toxic to fish and daphnids [104]. However, it has been proven that *N*-butyl benzenesulfonamide has neurotoxic effects on New Zealand rabbits. This compound is a plasticiser used in polyamide manufacture. Dose-dependent motor dysfunction in this kind of rabbits has been observed due to the intake of this compound [105]. Overall, toxicity assessments of benzotriazole, benzothiazole and benzenesulfonamide derivates toward to humans need additional tests because the information currently reported is still limited, taking into account the high production volume and everyday usage of these compounds.

Most benzotriazole, benzothiazole and benzenesulfonamide derivates are highly polar compounds because they are small compounds with polar groups embedded in their structure. Thus, their octanol-water partitioning coefficients (K_{ow}) are usually below 2 as Log P values for benzotriazoles and benzothiazoles, and close to 1 or lower for benzenesulfonamide and toluenesulfonamide compounds [106]. Therefore, they can reach surface waters and sewage easily and are only partially removed or not removed at all during conventional sewage treatment processes [107-109]. However, some of them are resistant to biodegradation, such as 4-methyl-benzotriazole. As a result, these classes of chemical substances are considered to be ubiquitous water contaminants. There are several studies on their occurrence in a range of matrices and they have been found to be present in surface water, ground water, tap water, sewage, sewage sludge, sediments and house dust, among others [99,107-113]. However, no maximum permitted levels have been regulated for these compounds in drinking water guidelines, except in the case of tolyltriazole (7 ng/L) and *para*-toluenesulfonamide (300 ng/L) in Australia [111] and Germany [99], respectively.

Information regarding the occurrence and analytical methods used for the determination of benzotriazole, benzothiazole and benzenesulfonamide derivates in environmental matrices is available in a review paper accepted for its publication in

the journal *Trends in Analytical Chemistry* and included in Section 1.2.3 of the present Thesis.

1.1.4. Nanoparticles

Nanomaterials and nanoparticles are natural or anthropogenic materials between 1 and 100 nm in size in at least one and two spatial dimensions, respectively. They can be spherical, tubular and irregularly shaped, and exist as fused, aggregated or agglomerated forms. Their nano-scale size confers them unique chemical and physical properties which are not observed by the same materials on a 'normal' scale [114-116]. These unique properties are mainly due to their large surface/area ratio, as the percentage of atoms at the nanomaterial surface becomes significant, which does not occur in bulk materials larger than one micron in size [117,118]. Thus, nanomaterials and nanoparticles exhibit high strength, high thermal stability, low permeability and high conductivity properties, among other unexpected properties. These remarkable properties stimulate the scientific community to focus their research on developing new uses and products related to nanotechnology. In addition, physical or chemical modifications of pristine nanomaterials, such as surface functionalisation, are commonly performed to modify or enhance their physicochemical properties. These kinds of nanomaterials and nanoparticles receive the prefix 'engineered', which is also used for the nanomaterials of anthropogenic origin.

Nanomaterials and nanoparticles can be classified as inorganic or organic (carbon-based) compounds and these two main groups are further sub-classified based on physicochemical properties, as showed in Figure 6. Inorganic nanomaterials include metal, metal oxides and quantum dots, such as silver, titanium dioxide and cadmium selenide nanoparticles. Meanwhile, organic nanomaterials are basically carbon structures which can have different spatial forms and, therefore, they may have a finite number of carbon atoms (fullerenes) or not (carbon nanotubes). Nonetheless, the list of both inorganic and organic nanomaterials is much more extensive and new engineered nanomaterials are produced every day.

Even though nanoparticles are considered a discovery of modern science, their use started centuries ago. The craftsmen of Mesopotamia (9th Century B.C.) used them for generating a glittering effect on the surface of pots and a distinct gold or copper coloured metallic glitter is still visible today in pottery from the Middle Ages and Renaissance. However, it was not until 1857 that Michael Faraday provided the

first scientific description of the optical properties of nanometer-scale metals. One hundred years later (1959), an embryonic idea of nanotechnology was discussed by Richard Feynman and, in 1974, the term 'nanotechnology' was first used by Norio Taniguchi, which became the starting point of exhaustive research into the fields of nanoscience and nanotechnology. As a result, Kroto *et al.* [119] reported the existence of a stable fullerene molecule (C_{60}) in 1985, and they were awarded the Nobel Prize in Chemistry in 1996 for their scientific contribution in the discovery of fullerene molecules. Since 2000, a few nanomaterials have started to be used in commercial products but new nanomaterials and their applications are still being developed.

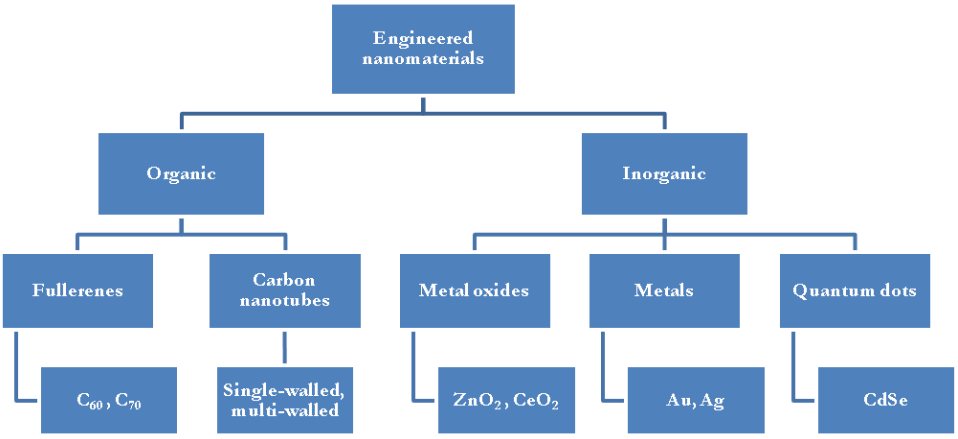


Figure 6. Nanomaterial classification according their physicochemical properties.

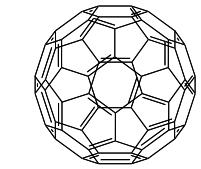
The most commonly used nanomaterial in consumer products is silver, followed by carbon (fullerenes and nanotubes), zinc oxide, silica, titanium oxide and gold. The global market value for nanomaterials is now estimated at €20 billion and is expected to grow to €2 trillion by 2015 [120-122]. The main uses of nanomaterials are very heterogeneous. For example, silver nanoparticles are used as anti-odour agents in sportswear or socks, due to their good antimicrobial efficacy. Gold nanoparticles are used in high technology applications, such as organic photovoltaic cells, as well as for drug delivery in biological and medical applications, based on their tuneable optical and electronic properties, by changing the size, shape, surface chemistry or aggregation state (e.g. Figure 7 shows the colour of an aqueous solution made with different sizes of spherical gold nanoparticles). Meanwhile, organic nanoparticles, such as fullerenes, are used in biomedicine, photovoltaic cells

and cosmetics. Fullerenes are pure carbon compounds and the most common are those between C_{24} and C_{84}. Furthermore, they are also functionalised for specific applications. For instance, C_{60} presents certain solubility limitations and so more soluble derivates (e.g. [6,6]-phenyl-C_{61}-butyric acid methyl ester) are synthesised to overcome a number of drawbacks observed in the fabrication of photovoltaic devices. As an example, Figure 8 shows the structure of different functionalised fullerenes. Another interesting organic nanoparticle is fullerol ($C_{60}(OH)_{24}$) [123], which is highly soluble in water, unlike C_{60} fullerene which is theoretically insoluble in water, as explained later.

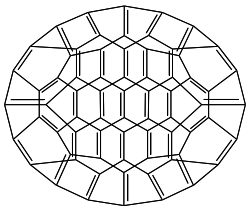
Figure 7. Aqueous suspensions of gold nanospheres from 5 to 100 nm in diameter (from nanoComposix®) [124].

Because of the extensive use of nanomaterials and the expected growth in their market in the coming years, it is necessary to address toxicological and risk assessment studies. The current knowledge and opinions of the EU Scientific and Advisory Committees and independent risk assessors agree on which nanomaterials are not dangerous *per se*, and which are similar to normal-size chemical substances. However, possible risks are related to certain nanomaterials and specific uses and, therefore, risk assessment studies needs to be performed on a case-by-case basis. To date, the toxicology of different nanomaterials has been tested in different stages of the food chain, starting with bacteria and finishing with terrestrial organisms and human and mammal cells. As expected, toxicological reports are highly dependent on the organism and nanomaterial involved, but it seems that metal oxide nanoparticles may display the worst toxic effects. For example, it has been observed that cerium dioxide nanoparticles damage the DNA of *Daphnia magna* (an aquatic invertebrate) [117], while titanium dioxide nanoparticles show different toxicity in a single cell organism, depending on the size of nanoparticles [125]. In contrast, gold nanoparticles or C_{60} fullerene did not show relevant toxic effects. However,

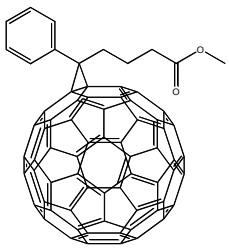
functionalised nanoparticles may have different toxicity because of their solubility and, as such, they may be capable of crossing different cell membranes.



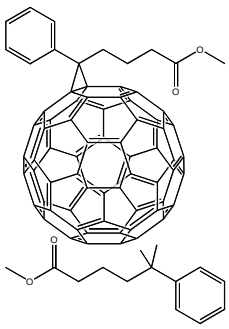
C_{60}



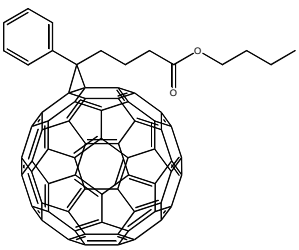
C_{70}



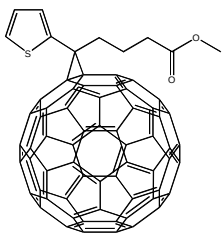
[6,6]-phenyl-C_{60}-butyric acid methyl ester



[6,6]-bis(phenyl)-C_{60}-butyric acid methyl ester



[6,6]-phenyl-C_{60}-butyric acid butyl ester



[6,6]-thienyl-C_{60}-butyric acid methyl ester

Figure 8. Structures of some fullerene compounds [118].

There is concern regarding nanomaterials as environmental contaminants due to their use in several products (cosmetics, clothes, photovoltaic cells, biomedicine, etc.) [126,127] and the amounts of these compounds produced (around 11 million tonnes per year worldwide) [114,115]. The US Environmental Protection Agency and EU agencies are now focused on assessing the environmental exposure of

nanomaterials and implementing environmental legislation. The ways in which nanomaterials can reach the environment are very diverse. The first route is through natural emission sources. This route is almost exclusively for fullerenes, as they originate in volcanic eruptions and forest fires, as well as through unintentional human activities like diesel exhaust. However, anthropogenic sources are the most important origin of environmental pollution, with wastewater discharges, manufacturing industries and erosion from composites being the main intrusion routes [22,116,128-137].

Most nanoparticles are insoluble in water but they form colloidal suspensions or aggregates that are stable in aqueous environments [138-143]. The most surprising case are aqueous fullerene aggregates (e.g., aqu/nC_{60}). The solubility of C_{60} in water is less than 10^{-9} mg/L but, when placed into an aqueous media, the fullerene molecule sits in an icosahedral water cluster because the size of fullerene molecule and the inner diameter of the water cluster are similar [144]. These water-fullerene clusters have acidic properties due to an acid-base reaction by electron-donor-acceptor complexation between the water and fullerene sp^2 carbons, resulting in a negatively charged surface which increases the solubility of C_{60} in more than eight orders of magnitude [145]. Furthermore, these single water-fullerene clusters may be aggregated between them originating bigger clusters or aggregates (Figure 9).

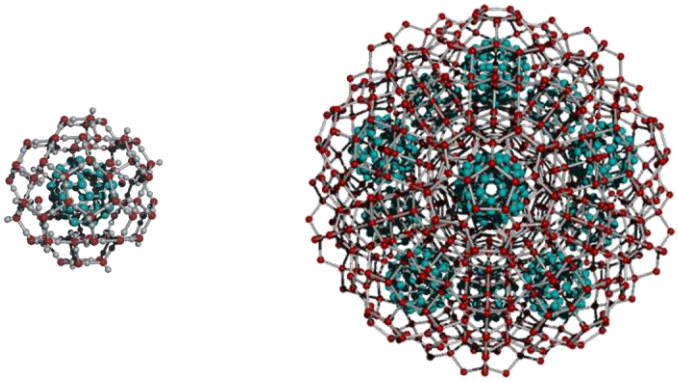


Figure 9. Single water-fullerene cluster (left) and aqueous fullerene aggregate (right).

As shown by different modelling studies, the presence of nanoparticles in environmental matrices is already accepted. However, most of the papers published regarding their determination focus on fullerene compounds, while reliable studies into inorganic nanoparticles have not yet been conducted, maybe because a number

of analytical challenges need to be overcome, such as distinguishing between nano-size and normal-size particles for inorganic compounds. Fullerene compounds are determined both in aqueous and solid matrices like sewage effluents or soils, at a broad range of concentrations, as shown in Table 5. The most widely expected concentrations of these compounds in environmental waters are in the range of a few ng/L. However, Farré *et al.* [146] found concentrations for C_{60}, C_{70} and N-methylfulleropyrrolidine in the μg/L range in some wastewater effluents from STPs located in highly populated and industrialised areas of Catalonia (Spain). In the other sampling sites far from industries and big cities, these compounds were also determined but at lower concentrations. Sanchís *et al.* [147] analysed river and sediment samples from nearby places and found C_{60} and C_{70} below ng/L levels. In addition, the same authors determined C_{60} in some soil samples from Saudi Arabia at concentrations ranging from 0.15 to 6.83 ng/g [147]. However, in most of the samples, these compounds were not detected.

Table 5. Occurrence of fullerene compounds in the environment.

Compound	Matrix	Conc. (ng/L, ng/g)	Country	Reference
C_{60}	River water	n.d.-7.9	Spain	[147]
	River water	98	Taiwan	[148]
	Industrial effluent	n.d.-130	Taiwan	[148]
	STP effluent	n.d.-19,100	Spain	[146]
	Sediment	n.d.-0.7	Spain	[147]
	Soil	n.d.-2.15	Saudi Arabia	[147]
C_{70}	River water	n.d.-1.2	Spain	[147]
	Industrial effluent	10-25	Taiwan	[148]
	STP effluent	n.d.-1,650	Spain	[146]
	STP influent	37	Taiwan	[148]
N-methylfullero-pyrrolidine	STP effluent	n.d.-65,900	Spain	[146]

As mentioned above, studies concerning the occurrence of inorganic nanoparticles in environmental samples are very scarce. Nonetheless, evidence exists that inorganic nanoparticles have their fate in sewage, surface water and sediments. For example, Benn *et al.* [133] found significant amounts of silver nanoparticles in washing water used in sock fabrics because of one of the major uses of silver is as an anti-odour agent in high-tech cloths. Therefore, the industrial wastes of these

fabrics are a potential source of silver contamination. In addition, Kim *et al.* [131] found silver sulfide nanoparticles in sewage sludge.

Most of the environmental studies into nanoparticles, either organic or inorganic, focus on their behaviour. Thus, the size, sedimentation, aggregation and stability of these compounds in water are some of the issues that are currently more widely researched. In general, it has been shown that natural organic matter could significantly affect the size and stability of nanoparticles in water and, therefore, increase their transport and bioaccumulation. However, much more research is still necessary to ascertain the real environmental concentrations of these compounds and, as a result, identify their behaviour and fate into environment.

UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
Pol Herrero Gil
Dipòsit Legal: T 1619-2015

1.2. Determination of emerging organic contaminants in environmental samples

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As mentioned above, emerging organic contaminants are a very large group of organic chemical substances which are present in the environment as a result of their anthropogenic use for several applications [3,7-9]. Since water should be considered as the live-based solvent, most of these chemical compounds are easily water soluble. Therefore, aqueous environmental matrices are the main points at which these compounds are likely to be found. Moreover, domestic and industrial sewage systems are the sites at which higher concentrations have already been found. Although there are thousands of compounds which may be considered to be emerging organic contaminants, their respective concentrations usually range from below parts per trillion to above parts per billion [13,34]. However, a level of parts per billion or trillion is the most likely range of concentrations expected for most emerging organic contaminants. Therefore, specific sample pretreatment techniques involving preconcentration and clean-up processes are necessary, before selective and sensitive analysis techniques for their determination in environmental matrices [21,34,97,115,149-157].

The extraction technique most commonly used for aqueous matrices is solid-phase extraction (SPE) which also allows the preconcentration of the analytes of interest. For solid matrices, such as sewage sludge or sediment samples, ultrasound assisted extraction (USAE), microwave assisted extraction (MAE) and pressurised liquid extraction (PLE) are the preferred extraction techniques, but high analyte preconcentration is not usually obtained.

After the extraction step, chromatographic techniques are usually required because of the similar physicochemical properties of the compounds belonging to the same family groups. In addition, efficient chromatographic separations reduce the matrix effect because of separation between the compounds of interest and undesired co-eluting matrix components. However, a universal chromatographic technique does not exist due to the great diversity of chemical compounds considered to be emerging organic contaminants. Thus, the most widely employed techniques are gas chromatography (GC) and liquid chromatography (LC). The choice between these techniques depends on the physicochemical properties of analytes, such as their volatility, thermal stability and polarity, among others. In addition to the separation technique used, powerful detection techniques in terms of sensitivity and selectivity are also needed. In this respect, mass spectrometry is the detection technique that has undergone the most remarkable development in recent years and, nowadays, it is an essential part of any analytical method to be applied for environmental analysis.

In the following sections, there is an overview of the different extraction and analysis techniques currently employed for the groups of compounds included in this Thesis. Most of the discussions and examples shown could be extrapolated for the determination of other emerging organic contaminants, since the general workflow for method development should be similar in many cases. However, specific considerations for each group of compounds need to be considered during method development and, therefore, a more in-depth discussion of glucocorticoids, polyether ionophores, benzotriazoles, benzothiazoles, benzenesulfonamides and fullerenes is presented.

1.2.1 Extraction techniques

As mentioned above, extraction techniques have been designed taking into account the physical state of the matrix, as they can be gaseous, aqueous, solid or semi-solid. The following sections focus on the extraction techniques related to aqueous and solid/semi-solid matrices, because these are the matrices studied in this Thesis. However, emerging organic contaminants are also present in all of the other matrices stated above.

1.2.1.1. Extraction techniques for aqueous matrices

Overall, the extraction techniques used for aqueous matrices are based on the partitioning equilibrium between the sample matrix (aqueous phase) and another phase, which can be a non-miscible solvent for liquid-liquid extraction (LLE) or a solid sorbent for solid-phase extraction (SPE). Until the 1980s, LLE was the most widely used technique for extracting different organic contaminants from environmental waters. However, conventional LLE often uses large amounts of organic solvents, resulting in analyte dilution and the formation of emulsions. Moreover, this technique is not suitable for the most polar compounds unless a previous derivatisation step is performed. An example of the difficulties of extracting polar compounds by conventional LLE extraction is reported in a paper for determining 1-H-benzotriazole in aqueous matrices [158]. To obtain recoveries around 80%, the acetylation of the target compound was necessary before its extraction with toluene. Thus, LLE methods are only used for hydrophobic compounds such as fullerenes, for which LLE is one of the most commonly used techniques, providing recoveries above 90% using toluene and adding a salt, mainly sodium chloride, to the aqueous phase [136].

In recent years, LLE has been revised and upgraded to new versions of this technique by miniaturisation of the process, which overcomes some of the related drawbacks mentioned above. Thus, in-vial LLE, single-drop microextraction (SDME), hollow-fibre microextraction (HFME) and dispersive liquid-liquid microextraction (DLLME) have been developed and applied for the determination of different emerging organic contaminants in environmental waters. Qin *et al.* recently developed a supramolecular solvent-based vortex-mixed microextraction (SS-BVMME) [159] and ionic liquid supported vortex-assisted synergic microextraction (ILSVA-SME) [160] to quantify trace amounts of glucocorticoids in surface waters. Supramolecular solvents (SUPRAS) are water-immiscible liquids formed by a sequential self-assembly of amphiphilic molecules at the molecular or nano scales arranged in a water matrix. They act as micelles or vesicles when critical aggregation concentration is reached. The most important property of SUPRAS is that their polarity changes in different zones, meaning that they can dissolve, concentrate and isolate the target compounds in a very specific way. For glucocorticoids, the supramolecular solvent was composed of the ionic liquid [BMIM]BF_4, n-butanol and water, obtaining recoveries between 88% and 103%. The sample volume was only 5 mL, since SS-BVMME is a miniaturisation technique, and the extraction time was less than 30 min. For ILSVA-SME, extraction principles are equal to SS-BVMME but, in ILSVA-SME, the solvent was composed of the water sample and the ionic liquid [BMIM]PF_6, a solution of Triton-X100 (0.05% v/v), which is a non-ionic surfactant. With this combination of solvents, recoveries above 97% were obtained for beclomethasone dipropionate, hydrocortisone butyrate and nandrolone phenylpropionate. As regards DLLME, a method using a ternary solvent has been reported for benzothiazole and benzotriazole compounds, obtaining recoveries between 67% and 97% using 9 mL of water sample. With this procedure, an extremely fast extraction of less than 5 min was obtained because the extraction solvents were dispersed into the water sample matrix as fine droplets, which conferred a large surface area for extraction.

Another technique related to DLLME was used by Chen *et al.* [148] for the determination of fullerene compounds in sewage and surface waters. They proposed ultrasound-assisted DLLME (UA-DLLME) for 10 mL of sample. The extraction procedure is similar to the procedure described above for conventional DLLME but, after dispersion of the extraction solvents into water sample, the cloudy solution is subjected to ultrasonication for 1 min. The authors obtained recoveries between 70% and 86%. SDME was applied for extracting monensin from soil, water and urine samples. The method was developed by Sekar *et al.* [161] and used

20 mL of sample (10% NaCl) and the solvent drop (1.5 μL) was composed of a mixture of chloroform with toluene (1:1, v/v). The recovery for monensin was close to 100% for surface water samples and close to 80% for soil and urine samples.

Before the explosion in the number of LLME techniques, solid-phase extraction (SPE) had already replaced LLE and, nowadays, it is still the most commonly used extraction technique for aqueous samples [150]. SPE emerged in the mid 1970s and has been widely used for extracting a broad range of compounds with different chemical properties from liquid samples. SPE is a sorption-based extraction technique in which the analytes present in aqueous sample interact with a solid sorbent on which they are retained. Afterwards, they are generally eluted using an organic solvent. The methodology of an SPE procedure is extensively known and can be found elsewhere in the literature. However, some considerations in terms of sample pretreatment should be pointed out, as they are specific to water analysis. Since the packed sorbents used for SPE consist of particles less than 100 μm in size, sample filtration is required before the loading step to prevent the sorbent from clogging. Usually, glass fibre filters (0.7-1.3 μm) or nylon membranes (0.22-0.45 μm) are used, but some authors also propose sand filtration [118]. This step is very important in sewage sample analysis because the raw sample has a large content of particulate matter. When a sample is filtrated, it is necessary to consider that some analytes may be retained on the filter by interactions, similar to the case of SPE materials. In addition, some analytes may also remain attached to particulate matter. However, the analyte amount attached to particulate matter is usually not considered, since the concentrations reported are just for the dissolved phase on its own. However, in some studies, particulate matter is included as a different matrix. For example, Asimakopoulos *et al.* [108] analysed both dissolved phase and particulate matter for benzotriazole and benzothiazole compounds and found several compounds in particulate matter, but usually at one or two orders of magnitude lower than in dissolved phase.

In the case of SPE, sorbent selection is the most important parameter to obtain efficient and reproducible extraction methods. The first sorbents developed were based on surface-modified silica particles with a wide variety of functional groups (C_{18}, C_8, CN, etc.). These kinds of sorbents are designed to retain non-polar compounds efficiently and so recoveries obtained for some of the smallest and/or polar organic contaminants were sometimes insufficient for the determination of these compounds in environmental samples. In addition, silica-based sorbents have a limited working pH range and the sorbent needs to remain wet during sample

loading in order not to lose retaining capabilities. However, the development of polymeric materials for SPE has solved these problems, since they can work in all pH ranges and the polymeric material remains wet for more time, which increases extraction reproducibility. Another advantage of polymeric materials is that they have a much higher surface area than silica-based sorbents and, therefore, they have more capacity, especially for hypercrosslinked polymers, which have a very large specific surface area (1,000-2,000 m^2/g). The most widely used sorbents for extracting EOCs from aqueous matrices are balanced hydrophilic/hydrophobic polymeric sorbents because they are more effective to retain a wide range of compounds with different polarities. One example of these sorbents is Oasis HLB from Waters®, which is a macroporous copolymer (800 m^2/g) made from a balanced ratio of divinylbenzene (hydrophobic) and *N*-vinylpyrrolidone (hydrophilic) monomers [162]. Thus, this combination of two classes of monomers with different properties makes this sorbent capable of retaining both polar and non-polar compounds.

For example, Liu *et al.* [40] compared Oasis HLB and Supelclean ENVI-C_{18} cartridges for extracting 28 steroids (five of which were glucocorticoids) from surface water and sewage. They found that the recoveries obtained for most of the compounds were lower than 80% using Supelclean ENVI-C_{18} cartridges, because the most polar compounds were not well-retained. Using Oasis HLB under optimised conditions, the authors obtained recoveries within the range of 91% to 119% for all of the compounds tested, except for 5α-dihydrotestosterone (143%). Vulliet *et al.* [44] also tested a broad range of SPE materials for extracting steroid hormones. Their study included Strata C18-E (C_{18} bonded silica), Strata-X (N-vinylpyrrolidone chemically-modified divinylbenzene polymer), IST ENV+ (styrene-divinylbenzene polymer) and Oasis HLB (*N*-vinylpyrrolidone-divinylbenzene copolymer). The best recoveries were obtained with the polymers that contained *N*-vinylpyrrolidone in its structure (Oasis HLB and Strata-X), with recoveries above 85% for glucocorticoids. For C_{18} bonded silica and styrene-divinylbenzene copolymer materials, the recoveries were below 50%. However, depending on the compounds of interest, similar recoveries may be obtained using either C_{18} bonded silica or polymeric sorbents. For example, Martínez-Villalba *et al.* [62] extracted eight coccidiostat compounds including five polyether ionophores from river water samples using a C_{18} sorbent, obtaining recoveries above 85%, except for robenidine compounds, which were only 60% recovered. Meanwhile, Kim *et al.* [70] and Cha *et al.* [78] obtained recoveries in the range of 83% to 123% for three polyether ionophores (included in a study by Martínez-Villalba *et al.*) using

the Oasis HLB sorbent. As can be seen, the results using both kinds of sorbents were very similar in this case.

However, for extracting non-polar compounds from water samples such as fullerenes, balanced polar/non-polar polymeric sorbents are not appropriate. For example, Wang *et al.* [163] used a Strata SDB-L cartridge (styrene-divinylbenzene polymer), which is suitable for retaining hydrophobic and aromatic compounds, for the extraction of fullerenes from water samples. However, C_{18} sorbents are the most commonly used in this case [118,164], as reported by Kolkman *et al.* [118]. The authors tested C_{18}, C_8 and CN silica-bonded phases for extracting functionalised fullerene compounds from water samples and obtained similar recoveries (70% to 120%) for C_{18}, end-capped C_{18} and C_8 sorbents, which were significantly better than those obtained with CN phase (40% to 75%).

In recent years, the development and use of polymeric sorbents with ionic-exchange moieties has increased. This kind of sorbents, known as mixed-mode, are characterised by having a polymeric structure modified with ionic groups, which adds ionic interaction with analytes to the typical interactions with the polymeric skeleton. Depending on the ionic group, they can be divided in cationic and anionic sorbents, and each one can also be subdivided into strong or weak mixed-mode sorbents. Thus, mixed-mode strong ion exchange sorbents are based on functionalised polymers with ionic groups such as quaternary amines (pK_a >18) or sulfonic groups (pK_a <1), and mixed-mode weak ion exchange sorbents are made by the addition of weak acids and bases to the polymeric structure. One of the advantages of this kind of sorbents is that elution can be performed sequentially using different pHs and solvents. Therefore, more selective and sensitive methods can be obtained by highly specific washing steps, which cannot be performed with conventional polymeric sorbents. These unique features of mixed-mode sorbents have been used in multiresidue analytical methods. For example, Kasprzyk-Hordern *et al.* [165] developed an analytical method by using an Oasis MCX strong cation-exchange mixed-mode polymeric sorbent to determine over 50 compounds in environmental water samples including multiple classes of pharmaceuticals, personal care products and illicit drugs. However, mixed-mode sorbents have rarely been applied to the compounds studied in this Thesis. Tölgyesi *et al.* [39] tested an Oasis MAX strong anion-exchange mixed-mode polymeric sorbent for the extraction of corticosteroids, androgens and progesterone from river water samples, obtaining good recoveries for corticosteroids. However, its use was discarded because it did not retain androgens well. Strong cation exchange (MCX) and weak cation exchange

(WCX) mixed-mode sorbents have also both been tested by Song *et al.* [80] for extracting monensin from river water, but better results were obtained using Oasis HLB instead of mixed-mode sorbents in this case.

As explained previously, mixed-mode sorbents are more suitable for ionic compounds but they can also be used for neutral compounds, thanks to hydrophobic interactions with its polymeric structure. Thus, it is possible to perform a sequential elution of these neutral compounds, which can provide less complex extracts because matrix components can be retained in the sorbent by ionic interactions under specific elution conditions. This feature is especially interesting for sewage analysis by LC-MS based methods in which the matrix effect is one of the major drawbacks [166-169]. Since sewage is composed of large amounts of fulvic and humic substances, their selective retention into the sorbent (meaning non-elution) during elution of the compounds of interest should be performed if the analytes are neutral and a mixed-mode strong anion exchange sorbent is used. This is because humic and fulvic substances have several carboxylic acid groups in their structure and, therefore, they are not eluted using non-acidic elution solvents. This effect is reported by Carpintero *et al.* [170] for the determination of several benzotriazole and benzothiazole compounds in surface water and sewage samples.

Other extraction techniques based on solid-phase extraction have emerged during the last decade. Examples of this include solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE). In the former case, the sorptive material is attached to a needle, generating a fibre where the analytes are retained. The latter technique is based on a magnetic stir bar covered with the sorptive material. These techniques have been applied for the determination of musk fragrances [171-173] and UV filters for SPME and some pharmaceutical compounds for SBSE, since slightly more polar materials are now available [174-176]. For the compounds included in this Thesis, the extraction techniques stated above have only been applied for extracting benzotriazoles, benzothiazoles and benzenesulfonamides. To the best of our knowledge, they have never used for the other compounds included in this Thesis. Thus, SPME has been used once by Naccarato *et al.* [177] for extracting benzotriazoles, benzothiazoles and benzenesulfonamides simultaneously from environmental waters, obtaining satisfactory recoveries for most of the compounds tested. Meanwhile, SBSE has been applied in a couple of papers for extracting benzotriazole and benzothiazole compounds. Nonetheless, the recoveries and preconcentration factors obtained using SPME and SBSE are far less than those obtained using conventional SPE

methods [174,175,178] because they are extraction techniques based on equilibrium processes.

1.2.1.2. Extraction techniques for solid matrices

Extraction techniques for solid matrices are based on partitioning analytes between the sample matrix (solid) and a liquid phase, which is usually an organic solvent. Generally, some sample pretreatment is needed before the extraction process. This includes freezing, lyophilisation and homogenization and, as such, the amount of analyte refers to sample dry weight. Classical extraction techniques for solid matrices include shaking and Soxhlet extraction. New extraction techniques which provide more efficient extraction and consume less solvent and time have replaced classical techniques, but these older techniques are still used in some analytical methods. For example, Sun *et al.* [81] and Kim *et al.* [70] used classical solvent extraction for the determination of polyether ionophores in soil and sediment samples, obtaining recoveries above 93% and 51%, respectively. Sun *et al.* [81] probably obtained better recoveries because they combined solid-liquid extraction (SLE) with LLE (methanol and hexane), while Kim *et al.* [70] only used an ammonium hydroxide buffer for SLE. Moreover, solvent extraction has also been used for the determination of pristine fullerene in sediments using toluene [163]. However, large amounts of solvents (40 mL twice) and time (60 min twice) are necessary for obtaining recoveries above 96% for C_{60}.

Environmental solid samples include sediments, soils and sewage sludge. They are very complex matrices, as a lot of compounds are present in them and, therefore, efficient and selective techniques are required. Nowadays, the latest and most widely used extraction techniques are ultrasound-assisted extraction (USAE), microwave-assisted extraction (MAE) and pressurised liquid extraction (PLE). However, most of the extraction methods for solid matrices are not very selective and so certain clean-up steps are performed during or after extraction to increase the selectivity of the overall extraction process, such as SPE, which is one of the most commonly used.

USAE uses the energy of ultrasound waves to increase the contact between the solid and liquid phases, thereby accelerating the extraction process compared to classical techniques. Moreover, sophisticated equipment is not required. For example, Liu *et al.* [40] and Fan *et al.* [41] determined some glucocorticoid compounds in sewage sludge using USAE. The authors used pure ethyl acetate and

a mixture of methanol and acetone (1:1, v/v), respectively. Using USAE, the extraction times were between 10 and 15 min and solvent consumption was around 10 mL. However, two or three extraction cycles were necessary to obtain recoveries in the range of 60% to 70% in [41] and in the range of 58% to 136% in [40]. USAE has also been widely applied for fullerene extraction from soil and sediment matrices. Sanchís *et al.* [147] and Carboni *et al.* [179] extracted fullerene compounds from the aforementioned matrices using toluene as the extraction solvent, obtaining recoveries between 50% and 70% for several functionalised and non-functionalised fullerenes in soil matrices [179], and between 79% and 108% for non-functionalised fullerenes and below 60% for functionalised fullerenes in sediment matrices [147]. USAE has been less employed for extracting the other compounds included in this Thesis. However, Zhou *et al.* [76] developed a multiresidue method for sludge, manure and sediment analysis and included three polyether ionophores in the study. The extraction was performed with 10 mL of acetonitrile and 10 mL of citric acid buffer in an ultrasonic bath for 15 min. The recoveries obtained in sediment samples were in the range of 70% to 122%, but were lower than 10% for sludge and manure. Benzotriazole and benzothiazole compounds were also determined in sewage sludge using USAE, with recoveries above 90% for most of the compounds included in the study [108].

PLE is based on the combination of high pressures (500-3,000 psi) and temperature (50-200ºC), without reaching the critical point of the solvent. These high pressures enhance the solubility, diffusion and desorption of the analytes from the matrix and, therefore, fast and efficient extraction methods are achieved. PLE is also known as pressurised fluid extraction (PFE), enhanced solvent extraction (ESE), high pressure solvent extraction (HPSE) and accelerated solvent extraction (ASE), which is the trademark name used by Dionex. PLE and ASE are the most commonly used terms. As in the case with MAE, PLE uses low volumes of solvent and semi-automation of the extraction procedure is possible. Since the solvent is automatically introduced into the extraction cell, different extraction cycles or sequential extractions using different solvents can be performed. Nowadays, PLE is one of the most extensively used extraction techniques for analysing solid and semi-solid environmental matrices because it is compatible with most of the solvents currently used for solid-liquid extraction and it provides more exhaustive extractions than other less sophisticated techniques [180]. For example, Gineys *et al.* [181] used PLE to determine trace levels of steroid hormones and corticosteroids in soil. The authors used a mixture of methanol and acetone (1:1, v/v) at 80ºC for 5 min and two cycles obtaining recoveries between 45% and 100% depending on the

compound. Additionally, Pérez-Carrera *et al.* [52] developed a method using PLE for the determination of 32 human and veterinary pharmaceuticals in soil and sediment samples. In this study, the glucocorticoid prednisolone was included and recoveries around 100% and 40% were obtained for this compound in soil and sediment, respectively. The extraction conditions were 80ºC, 1,500 psi, 5 min and 5 cycles, using a mixture of methanol and aqueous ammonia solution 0.1 M (1:1, v/v). The authors suggested that the higher organic carbon content and lower grain size of sediments compared to soils are the main reasons for the recovery differences between both matrices. PLE has also been used for extracting polyether ionophores from soil, sediment and manure [25,82,83]. The recoveries obtained are usually above 70% using different extraction solvents, such as methanol, methanol:acetone and methanol:water, as reported by Bak *et al.* [82]. Overall, an increase in extraction temperature has a strong negative effect on recoveries for certain polyether ionophores (monensin, salinomycin and narasin) if the temperature is higher than 70ºC. Moreover, slightly lower recoveries were observed in the same study for lasalocid acid and monensin in soil (71%) compared to sediment (83% to 94%) and manure (103% to 129%). In addition, benzotriazole compounds were extracted from sludge samples using PLE by Liu *et al.* [182] and benzothiazole compounds by Wick *et al.* [183]. PLE has not yet been applied for extracting benzenesulfonamide compounds and fullerenes from environmental solid samples. PLE also offers the option of performing some clean-up strategies during extraction. These include in-cell clean-up, in which the sample is mixed with an adsorbent material such as silica, alumina, Florisil or copper, to retain matrix interferences, and on-cell clean-up, in which a sequential extraction using different solvents with different polarities is performed to remove sample interferences prior to the extraction of compounds.

In addition, there are other extraction techniques which have been applied to the analysis of environmental solid samples but they have not yet been used for the compounds included in this Thesis. For example, MAE is based on the use of the energy of microwaves to heat the extraction solvent, which increases the kinetics of the extraction procedure. Thus, the extraction times are usually lower than USAE methods and less solvent is needed. MAE has generated great interest due to its capabilities and it has been tested for the extraction of a broad range of emerging organic contaminants, such as pesticides, polycyclic musk fragrances, antibiotics and hormones [169,184,185]. However, its application is still less common than USAE or PLE methods. One of the major limitations of MAE is the limited number of solvents which can be used, as the solvent has to absorb microwave energy, which only occurs in the case of polar solvents. Another extraction technique which has

been used for extracting solid matrices that is interesting due to its simplicity is QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction. This extraction technique has been widely used for the determination of pesticides in food matrices but other compounds can be extracted from other matrices using this technique. However, its application to environmental matrices is still limited to very few papers regarding the determination of pharmaceutical compounds [24] in sewage sludge and for the determination of polycyclic aromatic hydrocarbons [186] and chlorinated compounds [187] from soil samples. To induce solvent partitioning in QuEChERS extraction when environmental solid samples are analysed, little amounts of water are added, as these kind of samples are usually dry. In addition, QuEChERS extraction is usually followed by dispersive SPE (dSPE) in which a little amount of sorbent material (primary-secondary amine (PSA), C_{18} or graphitized carbon black (GCB) is dispersed in the organic extract obtained during extraction. This sorbent must be able to retain matrix interferences without retaining the compounds of interest and it is a very easy and fast step for sample clean-up. Thus, the main advantages of this technique are that sophisticated equipment is not required and the consumption of solvent and time is low.

1.2.2. Separation techniques coupled to mass spectrometry

As mentioned earlier, gas chromatography and liquid chromatography are the most common separation techniques used for the determination of emerging organic contaminants. In this section, there is a presentation of the most relevant features of the separation techniques used and mass spectrometric detection for the determination of the compounds covered in this Thesis in environmental samples. Chromatographic techniques coupled to mass spectrometry for the determination of benzotriazole, benzothiazole and benzenesulfonamide derivates are discussed more in-depth in a review paper included at the end of this chapter and, therefore, this is not discussed in this section. In addition to chromatographic techniques, flow-field flow fractionation has also been used for fullerene analysis in the present Thesis and this technique is also discussed.

Liquid chromatography (LC) is the only chromatographic technique reported for the determination of glucocorticoids in environmental matrices because both their extremely low volatility and the thermal instability of the hydroxyacetone side chain at position C17 do not allow their determination by gas chromatography (GC). However, GC has been used to determine these compounds in other matrices, such as biological fluids and tissues, for doping control or food safety. For example,

Amendola *et al.* [188] used gas chromatography coupled to mass spectrometry (GC-MS) with a previous derivatisation step with trimethylsilyl for screening glucocorticoid compounds in urine, which increases their volatility and preserves chemical structure information for their quantification and confirmation. However, glucocorticoids are generally determined by LC techniques coupled to tandem mass spectrometry.

In the case of LC separations, reversed-phase columns are the most widely used columns for glucocorticoid separation, with C_{18} stationary phases being the most common choice [36,37,40-42,181]. As well as these columns, certain authors have used other reversed-phase stationary phases. For example, Tölgyesi *et al.* [39] used a Supelcosil ABZ+ PLUS (Supelco) column to separate six corticosteroids, four androgens and progesterone for river water analysis. This column has an alkyl-amide stationary phase for increasing the retention of more polar compounds. In addition, less peak tail is obtained for the most reactive compounds with silanol groups on the silica particles. Other authors who have used different column chemistry for corticosteroid separation include Kitaichi *et al.* [43]. The authors tested C_8, C_{18} and C_{30} stationary phases and chose the C_{30} column because it provided good separation of the selected compounds in less than 15 min. However, an extended discussion regarding the chromatographic differences observed was not reported. As extracted from the data presented by the authors, the elution profile was the same for the three columns tested.

With respect to the mobile phase, either methanol [37,39-41] or acetonitrile [36,42,43,181] were used as the organic modifier, with water acidified with either formic or acetic acid usually being used for the aqueous phase. However, pure water [42] and ammonium acetate [39] were also used.

One of the major pitfalls of the separation of glucocorticoid compounds is that most of these compounds have a very similar structure and, therefore, their complete chromatographic separation is difficult. Moreover, there are certain isomeric compounds, such as betamethasone and dexamethasone, which are epimers and, as such, they are only differentiated in one stereo-position. Usually, the separation of these two compounds is skipped and they are quantified together or only one of them is chosen. However, this latter option is not accurate because their chemical similarity does not allow their individual determination if complete chromatographic separation between them is not obtained.

Mass spectrometry detection of glucocorticoids is usually performed by tandem mass spectrometry using triple quadrupole analysers (QqQ), as can be seen in the papers regarding their determination in different matrices, especially in environmental samples [37,39-43,181]. However, high resolution mass spectrometry using a linear ion trap Orbitrap hybrid mass analyser (LTQ-Orbitrap) has also been used by Schriks *et al.* [36]. For coupling liquid chromatography to mass spectrometry, an electrospray interface in either positive or negative mode is the most common option for these compounds due to their polar characteristics. Although positive ionisation is dominated by $[M+H]^+$ species, mass spectrum obtained for these compounds in negative ionisation mode depends on the mobile phase additive because of the formation of adducts. Thus, formate adducts $[M+HCOO]^-$ are observed if formic acid is used and acetate adducts $[M+CH_3COO]^-$ are observed if acetic acid is used. This specific feature has led some authors to suggest that negative ionisation is more selective than positive ionisation and, therefore, more suitable for their determination in complex matrices [189]. In addition, loss of water molecules by in-source fragmentation has been observed in positive mode for glucocorticoids, which may support the previous assertion. Chang *et al.* [37] determined cortisol, cortisone, dexamethasone, 6α-methylprednisolone, prednisolone and prednisone in sewage and river water by ultra-high performance liquid chromatography-electrospray-tandem mass spectrometry (UHPLC-(ESI)MS/MS) in negative mode. The authors use the transition $[M+HCOO]^-$ to $[M-H-CH_2O]^-$ for the quantification and $[M+HCOO]^-$ to $[M-H]^-$ for the confirmation. These transitions are common for all of the aforementioned glucocorticoids and these product ions are generated by the sequential loss of neutral formic acid and formaldehyde. In addition to electrospray ionisation, Tölgyesi *et al.* [39] use atmospheric pressure chemical ionisation (APCI) in negative mode to determine glucocorticoids. They use $[M+CH_3COO]^-$ as precursor ions for the determination of prednisolone, cortisol, dexamethasone, flumethasone and triamcinolone acetonide and $[M-H]^-$ for triamcinolone. The authors obtained LODs in the sub-ng/L level for river water, which are similar to those reported in other studies using electrospray ionisation.

The second group of compounds studied in this Thesis is polyether ionophores. As in the case of glucocorticoids, polyether ionophores are only determined in environmental matrices by LC-based methods. Due to their high molar mass, these compounds have very low volatility and, therefore, GC is not a suitable technique for their determination. The detection of polyether ionophores after LC separation is often achieved by tandem mass spectrometry methods, using either QqQ or ion

trap (IT) analysers. There is an interesting review by Hansen *et al.* [190] that focuses on analytical aspects for the determination of ionophores in environmental matrices. This review reports both extraction techniques and LC-MS/MS methods, and summarises the most relevant information regarding mobile phase composition and additives up to 2009, as well as mass spectrometry ionisation and fragmentation for these compounds.

LC separation of polyether ionophores are usually obtained with C_{18} reversed-phase columns due to their hydrophobic character [62,70,79,83,191]. The separation of the six most widely investigated polyether ionophores (lasalocid acid, monensin, maduramicin, narasin, nigericin and salinomycin) is not difficult, but their complete separation can take a long time because they are highly retained in C_{18} columns. To overcome the issue of the long analysis time, liquid chromatographic methods for polyether ionophores typically started at 50% organic mobile phase composition or above to reduce the analysis time. However, this reduction of analysis time by using starting conditions with high organic composition makes the separation of these compounds difficult. During their method development for the determination of four polyether ionophores in environmental matrices, Sun *et al.* [81] concluded that a mobile phase of water and methanol in combination with a C_{18} stationary phase is not capable of separating narasin and nigericin compounds. The authors propose the use of polar-embedded reversed-phase column (based on an alkyl chain modified with an amide group) in combination with a ternary mobile phase containing water, methanol and acetonitrile, which allows the chromatographic separation of the aforementioned compounds. However, the separation of narasin and nigericin can be obtained using acetonitrile instead of methanol with a C_{18} column, as reported by Martínez-Villaba *et al.* [62] in a paper which presents a method for the simultaneous determination of nine coccidiostats in river water (five of which were polyether ionophores). However, the use of acetonitrile results in the co-elution of monensin and lasalocid acid. Therefore, the use of ternary mobile phases seems to be the most promising option for the separation of polyether ionophores. With respect to mobile phase additives, 0.1% of formic acid in aqueous phase is the most common option [62,70,79,81] but 100 mM buffer of ammonium acetate has also been used [83,191].

As mentioned previously, detection of polyether ionophores is usually performed by tandem mass spectrometry operating in selected reaction monitoring (SRM) mode. Different mass analysers have already been used for determination in environmental matrices, with QqQ and 2D and 3D ion traps being the most widely

used. However, QqQ is more suitable than IT for environmental analysis because better sensitivity, instrumental detection limits and linear range are obtained [190]. In addition, time of flight (TOF), QTOF, LTQ-Orbitrap and Fourier transform ion cyclotron resonance (FTICR) mass analysers have also been used for structure and fragmentation pattern elucidation of polyether ionophores [62,190,192]. Due to the unique properties of polyether ionophores in terms to forming stable alkali complexes with sodium, electrospray ionisation in positive mode was the most commonly used ionisation interface because of the high abundance of the pseudo-molecular sodium adduct ion $[M+Na]^+$ obtained in MS spectra, which can be used as a precursor ion for MS/MS analysis. Since polyether ionophores have a carboxylic acid group in their structure, negative ionisation may be plausible for these compounds, but an LC-MS based method has never been optimised under these conditions. This is probably because a neutral compound is generated during ionisation, due to the complexation of a sodium atom by the polyether ionophore compound. Only lasalocid acid has been determined in negative mode because it is the most acidic polyether ionophore [77]. Besides electrospray ionisation, positive atmospheric pressure chemical ionisation has been proposed as a suitable ionisation technique for polyether ionophores by Schlüsener *et al.* [83]. The authors use APCI because this ionisation technique is less vulnerable to matrix effects than ESI, which is a very important factor for highly complex matrices. In this study, pseudo-molecular ammonia adduct ions $[M+NH_4]^+$ were observed because a 100 mM aqueous mobile phase was used for LC separation. However, more extensive studies have not been reported regarding the use of APCI and/or comparing APCI and ESI ionisation for polyether ionophores.

Fragmentation patterns of polyether ionophores are well elucidated because several papers have focused on this issue [192-195]. Lopes *et al.* [192,194] proposed the complete fragmentation pathways for monensin A and B, and lasalocid acid. The product ions observed were produced via Grob-Wharton fragmentation, pericyclic rearrangements and various neutral losses, as proposed by the authors, based on accurate mass measurements. For MS/MS measurements for quantification purposes, losses of multiple water molecules and carbon dioxide (neutral loss of carboxylic acid) are usually chosen because they are the most intense product ions obtained.

Apart from LC-MS based methods, Sekar and Wu [161] developed a quantitative method for the determination of monensin in soil, water and urine matrices by single-drop microextraction followed by direct analysis by atmospheric pressure

matrix-assisted laser desorption ionisation (MALDI)-mass spectrometry. The proposed method is simple, fast and efficient but the LODs obtained using MALDI-MS were in the range of μg/L for surface water samples, which are not low enough for application.

The last group of compounds discussed in this section are fullerenes. Once more, liquid chromatography is the preferred technique for fullerenes separation and either UV-Vis or MS have been used for their detection, due to the excellent response obtained with both techniques. However, MS provides better sensitivity and selectivity than UV-Vis. As mentioned in the introduction, fullerenes are present in environmental waters in aggregated form known as aqu/nC_{60} for C_{60} fullerene, for instance. It is suspected that the toxicity of fullerenes and other nanoparticles may be related to their size and, therefore, it is important to determine the size of these aggregates subject to environmental exposure. LC methods are not capable of providing this information and only the total concentration of fullerenes can be obtained. To overcome this limitation of LC methods, field-flow fractionation (FFF) sub-techniques (especially asymmetrical flow field-flow fractionation (AF4)) have gained interest for size determination, with a number of papers focusing on their use for fullerenes. As mentioned earlier, this technique is also used in this Thesis and so their principles and published applications on fullerene fractionation are reported at the end of this section.

In terms of LC methods, several papers on fullerene determination in environmental matrices have been published during the last few years. Usually, they focus on the determination of single C_{60} or multiple-functionalised C_{60} and C_{70} fullerene. For their separation, two different kinds of stationary phases have been used. These are either C_{18} [146-148,164,196,197] or the Cosmosil™ Buckyprep column [118,179,198], which has a stationary phase of pyrenyl-propyl functionalised silica. This stationary phase enhances the retention of fullerenes as a result of the large ligand that interacts with the aromatic structure of fullerenes [179]. In addition, Wang *et al.* [163] used a reversed-phase column with pentabromobenzyl group bonded silica packing material (Cosmosil™ 5PBB), which provides unique selectivity for structurally similar compounds, utilizing the dispersion force interaction. The dispersion force interaction makes it useful for the separation of structural isomers differing only by a double bond. However, these authors only determine C_{60} fullerene and so the specific properties of this stationary phase for fullerene separation has not been evaluated. The elution of these compounds which are strongly retained in reversed-phase columns due to the highly hydrophobic

character of fullerenes needs to be used with non-polar solvents. Thus, mobile phase rich in toluene (50-75%) is always used [118,196] and sometimes pure toluene. In separation based on C_{18} columns, methanol or acetonitrile are the polar components of mobile phase when a mixture of fullerenes is analysed. In the case of the Buckyprep column, either isocratic separation using pure toluene [198] and gradient elution separation starting at 25:75 (vol%) of acetonitrile:toluene have been proposed [118,179]. The latter separation is better for functionalised fullerenes, which is useful if the detection is performed by UV-Vis instead of MS. As well as the better separation obtained, methanol is usually added to the mobile phase because it enhances the ionisation during electrospray ionization. This enhancement can also be obtained by the post-column addition of methanol [118].

As regards mass spectrometry determination, fullerenes are ionised in negative mode, with ionisation performance using electrospray ionisation, atmospheric pressure chemical ionisation and atmospheric pressure photoionisation having been studied in a couple of papers [196,197]. These two studies, conducted by Núñez *et al.* [196] and Li *et al.* [197], demonstrated that the best performance for fullerenes was achieved with the APPI interface. This may be attributed to the high aromaticity of fullerenes and the fact that the use of toluene as the dopant improves the ionisation in the APPI interface. As explained above, toluene is one of the components of the mobile phase in all of the separation methods reported and, therefore, post-column addition of toluene is not necessary in this case. In contrast, post-column addition of methanol is required to enhance ionisation when ESI or APCI interfaces are used. In the study by Li *et al.* [197], similar sensitivity for C_{60} was observed using either APPI or APCI, whereas the sensitivity using ESI was much lower due to the formation of multiple adduct ions in addition to the fullerene molecular ion $[C_{60}]^-$, such as methanol adduct $[C_{60}+OCH_3]^-$, oxidised fullerene $[C_{60}+OH]^-$ and oxidised toluene adduct $[C_{60}+C_7H_7O_2]^-$. However, Núñez *et al.* [196] observed that APCI displayed bad performance for the ionisation of this family of compounds, which can be attributed to the different design of the interfaces used in the cited studies. Núñez *et al.* [196] also reported a very interesting study into the mass spectrometry of fullerenes using APPI interfaces. They explained the discrepancies observed in the isotopic pattern of fullerenes when APPI is used (m/z M+1 is more than 70% of m/z M for C_{60} when the theoretical abundance of m/z M+1 should be around 60%). The reason is that two isobaric ions contribute to m/z M+1 signal and they were assigned to $[C_{59}{}^{13}C]^{\cdot-}$ and $[C_{60}H]^{\cdot-}$ ions using a FTICR mass spectrometer operating at a resolving power of 400,000 FWHM.

Since fullerenes have a very specific structure only made by carbon atoms, fragment ions are not observed in tandem mass experiments and, therefore, SRM acquisition methods are not possible for these compounds. However, for functionalised fullerenes, SRM transitions from the parent ion to a pristine fullerene molecule can be achieved. These transitions are used for confirmation and not for quantification because the quantification of fullerenes is usually performed by SIM acquisition methods, if a QqQ instrument is used, or in full scan mode when other mass spectrometers such as IT or Orbitrap are used, because better sensitivity is obtained.

As mentioned previously, size determination of aqueous fullerenes aggregates and other nanoparticles in environmental samples is a very important issue due to the different toxicity and transport expected based on their size. Because conventional liquid chromatography methods do not provide size information, the use of field-flow fractionation (FFF), which can be considered as a family of chromatographic-like separation techniques, has dramatically increased in different research areas, such as pharmaceutical, biomedical and environmental science. In fact, the design of FFF instruments is very similar to an LC instrument because it is conducted using pumps, autosampler and detector, and the only difference is that FFF does not use a packed column for separation. In FFF, an open flat channel without stationary phase is used and this is where fractionation is achieved at a low-medium pressure. Since the first experiments of flow field-flow fractionation conducted by Giddings *et al.* in 1976 [199], a range of different sub-techniques of FFF have been developed to date. However, their use was limited for several decades by the lack of reliable instruments. These sub-techniques are named according to the nature of the perpendicular field applied. Flow field-flow fractionation (FlFFF), sedimentation field-flow fractionation (SedFFF) and thermal field-flow fractionation (ThermalFFF) are examples of this, but the most commonly used sub-technique nowadays is asymmetrical flow field-flow fractionation (AF4) which is a variation of FlFFF.

The fractionation principle of FFF is based on applying a perpendicular field to a main parabolic flow in an open flat channel, which results in the fractionation of particles as a result of the differences in their diffusion coefficient, due to Brownian motion and the different velocity across the channel height of the main parabolic flow. When this perpendicular field is a flow, the technique is known as FlFFF or AF4, depending on how the perpendicular flow (known as cross-flow) is generated. The main difference between FlFFF and AF4 is that the former has two permeable

walls (at the top and the bottom of the channel) and the latter only has one permeable wall (at the bottom). Both normal and asymmetrical flow field-flow fractionation principles are well reported in several papers and books [200-207]. In short, a porous membrane is placed at the bottom of the channel to retain the particles inside the channel and to allow the evacuation of the cross-flow applied. The cross-flow then pushes the particles against the porous wall (where the membrane is placed) and, due to their different Brownian motion, the smallest particles diffuse faster to upper layers inside the channel and, therefore, run to the detector faster than the bigger ones. This principle is true for particles between 1 and 1,000 nm because, in the case of bigger particles, another non-normal fractionation mechanism appears and the fractionation order is turned. Figure 10 shows a diagram of an AF4 channel and the fractionation principle. One of the most interesting features of FFFF is that particle size (the hydrodynamic radius) can be calculated from fractionation time using the Stokes-Einstein equation. However, multi-angle light scattering (MALS) or dynamic light scattering (DLS) detectors are usually placed on-line after the fractionation channel for size determination because theoretical calculations on AF4 systems with a fractionation using a cross-flow decay programme are very complex.

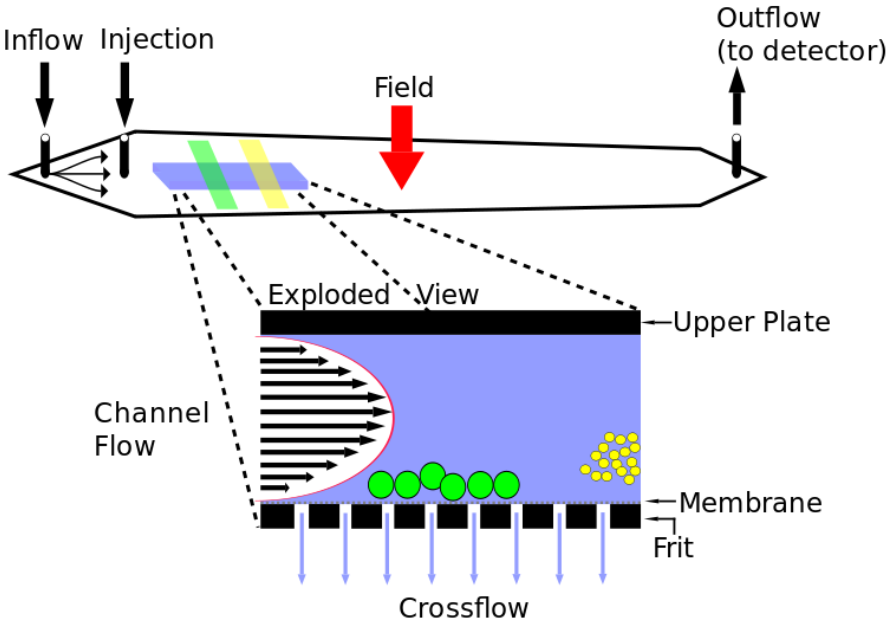

Figure 10. AF4 channel and fractionation mechanism.

AF4 techniques have already been used for fullerenes and other nanoparticles in the environmental field [139,203,208-216]. However, one of the major limitations of AF4 for fullerenes is the lack of detectors capable of determining their size distribution at the expected environmental concentrations, because MALS or DLS are not sensitive enough and preconcentration techniques cannot be used because they change the natural state of fullerenes in suspension [217]. Therefore, all of the studies regarding the behaviour of fullerene aggregates in environmental waters are related to lab-scale experiments at concentrations much higher (mg/L) than the real ones (ng/L). Isaacson *et al.* [208] propose AF4-DLS with off-line LC-MS analysis for the size distribution determination of aqu/nC_{60} at environmental concentrations. However, the method has not yet been applied to environmental samples. To date, reliable studies for the size determination of fullerene aggregates on environmental samples have not been published because detectors that provide size information are not sensitive enough.

Thus, on-line coupling of MS with AF4 for the determination of fullerenes (coupling for inorganic particles has already been performed by AF4-ICP-MS) seems to be a promising option to obtain size information at environmental concentrations of fullerenes. However, the high flow rates needed for AF4, compared with liquid chromatography, makes on-line coupling of AF4 with MS difficult. Only one paper using hollow-fibre FFF (HF5) has been published with on-line MS detection for proteins [218] and another using a chip AF4 (cAF4) for the determination of phospholipids [219]. These variations of FFF methods can be coupled more easily to MS because they need lower flow rates but their use in laboratories is not widespread.

1.2.3. Benzotriazoles, benzothiazoles and benzenesulfonamides in the environment: an overview of analytical methods and occurrence

UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
Pol Herrero Gil
Dipòsit Legal: T 1619-2015

BENZOTRIAZOLES, BENZOTHIAZOLES AND BENZENESULFONAMIDES IN THE ENVIRONMENT: AN OVERVIEW OF ANALYTICAL METHODS AND OCCURRENCE

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Abstract

Benzotriazoles, benzothiazoles and benzenesulfonamides are high-volume production chemicals widely used in industry and household every day. All these chemical substances are highly soluble in water and some have been shown to be resistant to biodegradation and/or have toxic effects. These chemical substances have already been classified as emerging organic pollutants, and some are considered ubiquitous water contaminants.

We present a review of the analytical methods currently employed for the determination of benzotriazoles, benzothiazoles and benzenesulfonamides in both aqueous and solid environmental matrices, as well as their occurrence in river water, sewage, soil, sediment and sewage sludge. Furthermore, we provide data and information on the removal and behavior of these compounds during sewage treatment.

Keywords: *Benzotriazoles; Benzothiazoles; Benzenesulfonamides; Environmental waters; Environmental solids; Sewage treatment plant; Extraction techniques; Liquid chromatography; Gas chromatography; Mass spectrometry.*

1 Introduction

A group of environmental pollutants currently being investigated is the high-volume production chemicals, which include benzotriazole, benzothiazole, benzenesulfonamide and their chemical derivates, among others. Due to their widespread use in various applications and everyday consumer products, these chemical compounds easily reach environmental water and solids. As a result of the evident pollution of the environment, the scientific community is striving to ascertain the current level of chemical pollution in different environmental matrices and therefore the specific analytical methods that need to be developed to determine a broad range of chemical pollutants in these matrices [1-5].

Benzotriazoles are heterocyclic compounds containing two fused rings and three nitrogen atoms, and the chemical formula of the parent compound is $C_6H_5N_3$ (Figure 1). One of the main features of benzotriazoles is that they can form a stable coordination compound with some metals, e.g. cooper, which confer anticorrosion properties [6]. Beside this, benzotriazoles are extensively used as corrosion inhibitors in some fluids like antifreeze formulations, cooling systems, hydraulic fluids or dishwasher detergents, among others. In addition, some derivates have specific chemical and biological properties which make them useful in pharmaceutical industry. The most common benzotriazoles are the parent compound benzotriazole, and tolyltriazole, which is a mixture of the isomers 4- and 5-methyl-1H-benzotriazole. Other classes of benzotriazole compounds are the derivates used as UV filters. This specific kind of benzotriazole derivate is not included in this review, because they are not commonly determined with the compounds included in this review and a specific review has recently been published by Montesdeoca-Esponda *et al.* [7].

Benzothiazoles are aromatic heterocyclic compounds consisting in a 1,3-thiazole ring fused to a benzene ring, and the chemical formula of the parent compound is C_7H_5NS (Figure 1). Benzothiazole derivates are widely used as vulcanisation accelerators on rubber, as biocides in paper and leather manufacturing, and also as anticorrosive agents in antifreeze formulations and photosensitisers in photography [8]. Their derivates are based on the substitution of a functional group at the methyne position in the thiazole ring. One of the most widely used derivate is 2-mercaptobenzothiazole as a vulcanization accelerator, while the parent compound is not widely used.

Benzenesulfonamides have a benzene or toluene ring with a sulfonamide group substituent. The formula of the parent compound is $C_6H_7O_2NS$, and their derivates are mainly *N*-substituted aliphatic compounds (Figure 1). These compounds are frequently used in several applications. For example, the parent compound benzenesulfonamide is used in the synthesis of dyes,

photochemical products and disinfectants; *para*-toluenesulfonamide is used as a plasticiser or as a fungicide in paints and coatings; *ortho*-toluenesulfonamide is used in the synthesis of artificial sweeteners like saccharin; and some *N*-substituted toluenesulfonamides are also used as plasticisers [9,10].

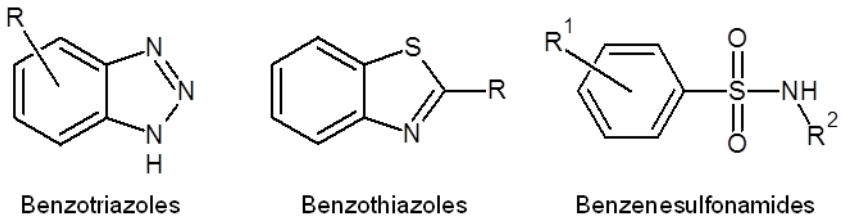

Fig. 1. Chemical structures of BTRs, BTs and BSAs.

All of these classes of chemical substances are highly soluble in water and some, like tolyltriazole, are resistant to biodegradation and/or present toxic effects [11]. Moreover, 1-H-benzotriazole is a suspected human carcinogen and has estrogenic effects in fish [12]. There is a lack of information on the toxicity of benzothiazole derivates, but acute aquatic toxicity has been reported [13]. *Para*-toluenesulfonamide has been shown to be moderately toxic, but due to the large amounts used, the Organisation for Economic Co-operation and Development (OECD) recommended additional tests [14]. These compounds have therefore already been classified as emerging organic pollutants, and some are considered ubiquitous water contaminants, which has led to the establishment of a maximum limit for tolyltriazole (7 ng/L) and *para*-toluenesulfonamide (300 ng/L) in the drinking water guidelines of Australia [15] and Germany [10], respectively. Due to their chemical properties, these compounds are highly mobile in aquatic systems and they are released to surface waters mainly by effluents of municipal and industrial sewage treatment plants, since their removal by conventionally sewage treatments is low [2,16]. As mentioned above, determining water quality and emission sources of chemical contaminants is a priority issue increased by the growing interest in reusing sewage effluents and sludge. Sensitive, selective and reliable analytical methods are thus necessary to determine the low concentrations of these contaminants in environmental matrices. This review provides an overview of the analytical methods employed from 2000 to the beginning of 2014 for the determination of benzotriazole, benzothiazole and benzene-sulfonamide derivates both in aqueous and solid environmental matrices. The review aims to cover the major challenges for the proper determination of these compounds. The current data available on their

occurrence in environmental waters and solids and their removal during sewage treatment is briefly discussed.

2 Analytical methods

Like other emerging organic contaminants, benzotriazole, benzothiazole and benzenesulfonamide derivates are usually found at concentrations of ng/L or ng/g in environmental waters and solids, respectively. Efficient extraction procedures followed by sensitive instrumental techniques are therefore necessary for their determination in environmental samples. The most commonly used extraction technique for these compounds is currently solid-phase extraction (SPE) for aqueous matrices [15,17-26], while different techniques are used when solid matrices are analysed, such as pressurised liquid extraction (PLE) [27-29] or ultrasound assisted solvent extraction (USAE) [30,31]. The analytical techniques applied after extraction are commonly chromatographic separations by either liquid (LC) [15,18-20,22,24-26,29,32-36] or gas chromatography (GC) [21,23,37-39] coupled preferably with tandem mass spectrometry, but UV-Vis or fluorescence detection have also been used in LC [40].

The current studies on analytical strategies for the determination of these emerging organic contaminants in environmental matrices are described in the following sections. First, the instrumental techniques applied for their determination are discussed. The extraction techniques either for aqueous and solid samples are also subsequently discussed. The most relevant publications on the analytical methods employed for the determination of benzotriazoles, benzothiazoles and benzenesulfonamides in environmental matrices are summarised in Table 1, and some of these are discussed in more depth in this review.

2.1 Instrumental analysis

2.1.1 Liquid chromatography

As can be seen in Table 1, the preferred instrumental analytical technique is either LC or GC, coupled with tandem mass spectrometry due to its high selectivity and sensitivity. However, LC has been more widely used due to the low volatility of these compounds. Several aspects of chromatographic parameters need to be assessed because of the high polarity of these compounds and the existence of isomeric species, such as 4- and 5-methyl-1-H-benzotriazole or *ortho*- and *para*-toluenesulfonamide. Thus, different columns have been used by different authors, with C_{18} [18,19,24,26,32,34,40,41] and phenyl [20,22,35,42] stationary phases the most used, but C_8 [33] and polar-embedded reversed-phase columns [26] were also tested. A chromatogram obtained by LC-(ESI)HRMS of a mixture of different benzotriazoles, benzothiazoles and benzenesulfonamides is showed in Figure 2 [26]. As expected from the

Table 1. Analytical methods reported for BTRs, BTs and BSAs in environmental samples.

Compounds	Matrix	Extraction technique	Instrumental analysis	LODs (ng/L, ng/g)	Ref.
BT, MeSBT, OHBT, BTR, 4TTR, 5TTR, MepTSA, pTSA, EtpTSA, EtmTSA, EtoTSA	River water and sewage	SPE; Strata-X	GC x GC-HRMS	5-112	[34]
BTR, 4TTR, 5TTR, XTR, ClBTR, BT, MeBT, NH_2BT, OHBT, SHBT, MeSBT, BSA, pTSA, EtpTSA	River water and sewage	SPME; polyacrylate	GC-MS/MS	0.1-7 500	[38]
BT, MeBT	Influent sewage	SBSE; PA Twister	TD/GC-MS	256	[36]
BT, NH_2BT, MeSBT, SO_3BT, SHBT, OHBT	Sewage	SPE; Oasis HLB	LC-MS/MS	25-420 (LOQ)	[24]
BTR, 4TTR, 5TTR, OHBTR, ClBTR, BT, NH_2BT, OHBT, MeSBT	River water and effluent sewage	SPE; Oasis HLB	LC-HRMS	1-10.0	[17]
BTR, 4TTR, 5TTR	River water and sewage	Direct injection or SPE, Oasis HLB	LC-MS/MS	10-25 (LOQ)	[27]
BTR, 4TTR, 5TTR	River water and effluent sewage	SPE; Oasis HLB	LC-MS/MS	0.2 (LOQ)	[15]
BTR, 4TTR, 5TTR	River water	SPE, C_{18}	GC-MS	-	[55]
BTR, 4TTR, 5TTR	Soil	USAE, DCM	GC-MS	-	[56]
BTR, 4TTR, 5TTR	Soil	LSE, ACN/water (1:1)	LC-MS/MS	2.2-3.1	[18]
BTR, TTR	River water	SPE; Oasis HLB	LC-MS/MS	3.0-8.0	[29]
NH_2BT, BT, MeBT, TCMTBT, MeSBT, SO_3BT, SHBT, OHBT	Sewage	Direct injection	LC-MS/MS	10.0-2 500	[23]

Table 1. (Cont.).

Compounds	Matrix	Extraction technique	Instrumental analysis	LODs (ng/L, ng/g)	Ref.
BT, MeSHBT, BTR, OHBT, 5TTR, XTR, SHBT	Sewage	SPE; Strata-X	GC-MS	25-562	[39]
BTR, TTR, OHBT, NH_2BT, XTR, SHBT, BT, MeBT, MeSBT	River water and sewage	SPE; Oasis MAX	LC-MS/MS	2-286 (LOQ)	[26]
BTR, 5TTR, ClBTR, XTR	River water and sewage	SPE; Oasis HLB	GC-MS/MS	14-47	[35]
	Sludge and sediment	PLE; MeOH/DCM (50:50)	GC-MS/MS	3.0-14.0	
BTR, OHBTR, TTR, XTR, BT, OHBT, MeSBT, NH_2BT	Sewage	SPE; Strata-X	LC-MS/MS	0.08-16.70	[19]
	Sludge	USAE and SPE; MeOH/water (1:1)	LC-MS/MS	0.04-12.50	
BT, MeSBT, SO_3BT, OHBT, MorBT	River water and sewage	SPE; HLB	LC-MS/MS	0.5-200 (LOQ)	[30]
	Sludge	PLE and SPE; MeOH/water (1:1)	LC-MS/MS	2.5-100 (LOQ)	
BTR, 5TTR	Sludge and sediment	LSE and SPE; MeOH and Oasis HLB	LC-MS/MS	0.07	[47]
OHBTR, BTR, 4TTR, 5TTR, ClBTR, XTR, NH2BT, OHBT, SHBT, BT, MeSBT	Influent sewage	DLLME; TBP	LC-Flu-UV	40-2 600	[21]
			LC-MS/MS	-	
BSA, pTSA, oTSA	River water and sewage	SPE; SDB	LC-MS/MS	10-20 (LOQ)	[28]

Table 1. (Cont.).

Compounds	Matrix	Extraction technique	Instrumental analysis	LODs (ng/L, ng/g)	Ref.
BTR, TTR	River water and sewage	SPE; Oasis HLB	LC-MS/MS	3.0-8.0	[57]
BTR, MeBTR, 4TTR, 5TTR	Effluent sewage and sludge	SPE; Oasis HLB	GC/IRMS	1 900-6 000	[6]
BTR, 4TTR, 5TTR, XTR, ClBTR, BT, NH_2BT, OHBT, MeSBT, BSA, pTSA, oTSA, MepTSA, EtpTSA	River water and sewage	SPE; Oasis HLB and Florisil	LC-MS/MS	1.0-20.0	[20]
BTR, 4TTR, 5TTR, XTR, ClBTR, BT, NH_2BT, OHBT, MeSBT, BSA, pTSA, oTSA, MepTSA, EtpTSA	Sludge	PHWE and SPE; Water and Oasis HLB and Florisil	LC-HRMS	0.25-25	[46]
BTR, 4TTR, 5TTR, XTR, ClBTR, BT, NH2BT, OHBT, MeSBT, BSA, pTSA, oTSA, MepTSA, EtpTSA	Sludge	QuEChERS; ACN and Z-sep+	LC-HRMS	0.5-10	[32]
BTR	Sewage	Derivatisation and LLE; Toluene	GC-MS	10 000 (LOQ)	[37]

relatively high polarity of these compounds, their retention factors in reversed-phase columns are low and methanol is therefore usually used as organic modifier for mobile phase in LC methods. However, the use of methanol does not provide any separation between the two tolyltriazole isomers, and as such most of the papers omitted their separation. The separation of tolyltriazole isomers can be obtained if acetonitrile is used instead of methanol as organic modifier for LC separations because a different chromatographic selectivity for these compounds is obtained [19,22,26]. The separation of benzenesulfonamide compounds by LC methods applied to environmental samples has only reported by Richter *et al.* [35] and

Herrero *et al.* [26]. The non N-substituted benzensulfonamide compounds (benzenesulfonamide and toluenesulfonamide isomers) have a low retention in reversed-phase columns and therefore, separating the two isomers is difficult. Richter *et al.* [35] obtained the complete separation of orto- and *para*-toluenesulfonamide by an isocratic elution with water/acetonitrile (9:1) using a C_{18} column (250 x 2.1 mm, 3.5 μm) but more than 35 min at a flow rate of 0.2 mL/min was necessary.

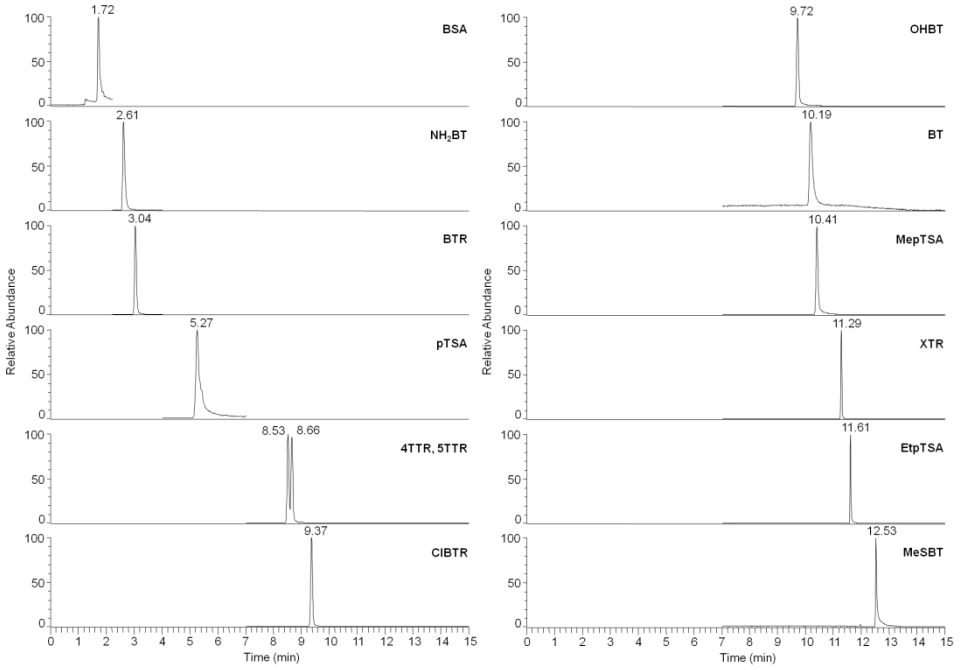

Figure 2. EIC chromatograms of a 250 μg/L standard mixture of BTRs, BTs and BSAs by LC-(ESI)HRMS from [36].

Herrero *et al.* [26] obtained a slight separation between the two isomers in 5 min working at a flow rate of 0.8 mL/min and using a C_{18} column (100 x 2.1 mm, 2.7 μm) due to the higher chromatographic efficiency provided by superficially porous particles. Comprehensive studies of the influence of the pH of the mobile phase on the chromatographic behaviour for these compounds are not reported because most of them do not present strong acid or basic properties. Some authors propose the use of ammonium acetate 1-10 mM [22,34] but acetic [26,40] and formic acid have also been used [19,20]. The selection of the modifier of the mobile phase is usually based on the ionisation efficiency obtained for some compounds when MS is coupled to LC rather than the

chromatographic separation itself. Briefly, 2-aminobenzothiazole and 2-mercaptobenzothiazole are not ionised when formic acid is used, and the addition of ammonium salts suppresses the ionisation of benzothiazole-2-sulfonic acid by ion-pairing reaction if the concentration is higher than 10 mM [34].

As mentioned above, liquid chromatography is usually coupled with mass spectrometry for the determination of these compounds. While spectroscopic determination by UV-Vis or fluorescence [40] are also a good option, their sensitivity and selectivity are not enough to achieve their determination at low concentrations in complex samples, as is the case for most of the environmental matrices.

Because BTRs, BTs, and BSAs are polar compounds, electrospray is the most commonly used interface for these compounds, providing good ionisation efficiency in positive mode for all of them, except for 2-hydroxybenzothiazole which ionises well in both modes [26] and benzenesulfonamide and toluene-sulfonamide, which only ionise in the negative mode [26]. Nonetheless, atmospheric pressure chemical ionisation (APCI) has been used by Wick *et al.* [25]. The authors compare ESI and APCI for a wide range of compounds, including some benzothiazoles and find that APCI provides limits of quantification (LOQs) that are 4-5 times better than ESI for benzothiazole and 2-(methylthio)benzothiazole in sewage influent samples, because the matrix effect is strongly decreased if APCI is used. Nonetheless, for other compounds such as 2-hydroxybenzothiazole, similar LOQs are obtained with both interfaces.

Tandem mass spectrometry using triple quadrupole analysers (QqQ) is the most reported technique used [18,22,24,34], but high resolution mass spectrometry (HRMS) based on FT Orbitrap analysers have also been used [19]. Nowadays, the best sensitivity on target analysis is provided by QqQ analysers operating in selected reaction monitoring (SRM). However, most environmental analysis methods often follow the guidelines of Directive 2002/657/EC [43], which requires that at least two SRM transitions for each compound should be monitored for a proper identification of target analytes. In the case of the compounds covered in this review, they are small molecules (especially for non-derivate compounds) and the second product ion needed for confirmation may therefore have very low abundance, which increases their limit of detection (LOD) and quantification (LOQ). As indicated in the aforementioned directive, if HRMS is used, only the precursor ion and one product ion are necessary for a proper identification of analytes due to its higher specificity.. Similar LODs and LOQs are therefore obtained for these compounds using either MS/MS or HRMS due to the low abundance of second SRM transition required in MS/MS analysis [36]. In addition, a decrease in method selectivity when MS/MS is used

needs to be assessed due to the low mass obtained for the product ions of these compounds [44].

One of the major issues for the analysis of environmental samples by LC-MS based methods is the matrix effect (either ion suppression or enhancement) which makes obtaining an accurate quantification difficult. To correct this, a matrix-matched calibration is often used in environmental analysis. However, the high environmental concentration of some compounds (e.g. BTR and TTR) makes using them impossible [22,26]. External calibration with standard solutions is thus often adopted for the quantification of BTRs, BTs and BSAs. When this option is used, the addition of surrogate compounds [17], mainly 5, 6-dimethyl-1H-benzotriazole (XTR), or isotopically labelled compounds [19] (e.g. BTR-d4) is mandatory to compensate for the differences in analyte response. Nonetheless, the most accurate option is standard addition calibration, but this is time consuming and laborious and is therefore rarely used.

2.1.2 Gas chromatography

Benzotriazole, benzothiazole and benzenesulfonamide compounds can also be determined using gas chromatography without any previous derivatisation step. However, the use of GC is less widespread than LC, as can be seen in Table 1, due to the difficulty to find a suitable GC column to separate a large number of these compounds. Fused silica columns coated with 5% diphenyl/95% polydimethylsiloxane [6,23,37,45,46] are the most used for the separation of some of these compounds. However, in a recent paper, this column has been used for the simultaneous determination of benzotriazoles, benzothiazoles and benzenesulfonamides by Naccarato *et al.* [37], but an extended discussion on GC method development was not included. Ionic liquid stationary phases have also been proposed [21] and for instance, Domínguez *et al.* [21] tested different ionic-liquid stationary phases for the simultaneous determination of benzotriazole and benzene-sulfonamide compounds, with very promising results for their determination due to their unique selectivity and lower column bleeding. A good option for the simultaneous separation of these three groups of compounds is the method developed by Jover *et al.* [17] using comprehensive two-dimensional gas chromatography (GC x GC) coupled with time-of-flight mass spectrometry. The study of Jover *et al.* [17] comprises the testing of different column polarities and moreover, the separation of tolytriazole and *N*-methyl-(*ortho*-, *meta*- and *para*-) toluenesulfonamide isomers is obtained.

Electron impact (EI) is the preferred ionisation technique for these compounds. However, EI (70 eV) is a hard ionisation and low mass fragments for BTR, BTs and BSAs are therefore obtained, decreasing the selectivity of MS measurements and

sensitivity for MS/MS methods as can be seen in the LOQs listed in Table 1.

Gas chromatography isotope ratio mass spectrometry (GC/IRMS) was also used for determining benzotriazole and its derivates by Spahr *et al.* [6]. These measurements are based on a compound-specific isotope analysis by comparing the ratios obtained by $\delta^{13}C$ and $\delta^{15}N$ in a combustion reactor. The conventional oxidation reactors used in IRMS are usually made of Cu/Ni/Pt, but the authors use a modified set-up with a Ni/Ni/Pt oxidation reactor due to the ability of benzotriazole derivates to form organometallic complexes with copper, which makes their determination impossible using conventional reactors. Unfortunately, the LODs obtained by GC/IRMS are far from useful in environmental analysis, since a preconcentration of more than 105-fold may be necessary for environmental aqueous samples [6].

2.2 Extraction techniques

2.2.1 Aqueous samples

Aqueous samples comprise a wide range of environmental matrices with significant differences in terms of their complexity. Sewage samples in general and influents in particular are thus the most complex matrices due to the large amount of organic matter and suspended material, and they are also where benzotriazoles, benzothiazoles and benzene-sulfonamides, among other environmental contaminants, are found at highest concentrations. Nonetheless, these compounds are also found in surface waters, ground water and tap water, as already mentioned. Analytical strategies for extracting benzotriazole, benzothiazole and benzene-sulfonamide derivates from aqueous samples are related to solid-phase extraction (SPE) but solid-phase microextraction (SPME), dispersive liquid-liquid microextraction (DLLME) and stir bar sorptive extraction (SBSE) have also been used in some cases. Before applying any of these techniques, sample filtration is mandatory to prevent clogging on the SPE cartridge and/or to remove the particulate matter from the aqueous phase. No special care is necessary to prevent analyte degradation, except for 2-mercaptobenzothiazole because the thiol group is easily oxidised in aqueous solution. Kloepfer *et al.* [47] propose adding glutathione solution to the samples to preserve it from oxidation, and Carpinteiro *et al.* [22] report that at least 5 mM concentration of glutathione in samples is necessary. Because of the concentration of some benzotriazoles, such as 1-H-benzotriazole and tolyltriazole, and some benzothiazoles, such as 2-hydroxybenzotriazole and methyl (thio)benzotriazole, is in the order of few μg/L in influent sewage, they can be also determined by the direct injection of samples without sample preconcentration. Weiss and

Reemtsma [20,34] thus propose the use of the direct analysis of some samples (after filtration) by injecting 60-100 μL in the LC-MS/MS equipment. The LOQs obtained were around 100 ng/L depending on the compound. Direct injection analysis is a very useful approach for monitoring studies in which a large number of samples need to be processed and time and cost are thereby reduced. However, the LODs obtained may not be enough depending on the matrix and compound and extraction and preconcentration techniques are therefore usually required.

As shown in Table 1, the preferred extraction technique is SPE. This technique provides a high sample preconcentration factor because large volumes of aqueous samples are used. These volumes are dependent on the sample's complexity and they are usually as follows: 50-500 mL for sewage; 100-1 000 mL for surface and ground water; and 500-1 000 mL for tap water.

Today, the most common SPE sorbents used for extracting these compounds are polymeric balanced polar/non-polar sorbents, which can retain more polar compounds than conventional C_{18} sorbents because of the high polarity of most BTs, BTs and BSAs (normally their $\log K_{ow}$ is below 2) [48]. Examples of these sorbents are Oasis HLB (*N*-vinylpyrrolidone-divinylbenzene copolymer) and Strata-X (*N*-vinylpyrrolidone chemically modified divinylbenzene polymer). Benzotriazoles are usually determined together with benzothiazoles in one single analysis, but some authors also determine benzenesulfonamide compounds in the same analysis. For example, Jover *et al.* [17] use Strata-X sorbent to extract three benzothiazoles, three benzotriazoles and four benzenesulfonamides from river water and sewage, obtaining recoveries between 68% (MeSBT) and 115% (5TTR) and LODs between 5 and 112 ng/L depending on the compound and the matrix. The authors use XTR and MeSMe as surrogates to correct the recoveries for benzotriazole and benzothiazole compounds, and benzenesulfonamide compounds, respectively. The use of XTR as a surrogate for BTRs and BTs is common in many papers because it is rarely found in environmental waters. However, some authors have reported its presence [23,26,49,50] and its use should therefore be revised.

Van Leerdam *et al.* [19] use Oasis HLB for the simultaneous determination of six benzotriazoles and four benzothiazoles in sewage, surface water and tap water. The authors tested different pHs and elution solvent mixtures for SPE but they did not find any significant effect on recoveries when the sample pH was modified. The recovery decreased at acidic pH only for the compound 2-aminobenzothiazole which has basic properties. As for elution solvent, pure methanol, acetonitrile and a mixture of both solvents were tested, with the mixture of solvents the best option, but significant differences were not

observed. Moreover, the authors tested 200 and 500 mg of sorbent, and 500 mg was much more effective to retain 2-aminobenzothiazole, which is the most polar compound. The recoveries were above 68% for all compounds, and 1-H-benzotriazole-d_4 was used as the internal standard to correct the differences in analyte response caused by the matrix effect.

Mixed-mode strong anion exchange sorbent (Oasis MAX) was also applied for extracting benzotriazole and benzothiazole derivates from aqueous samples.. As can be seen in Table 1, Carpinteiro *et al.* [22] used Oasis MAX for extracting BTRs and BTs in river water and sewage in a different procedure to the one initially designed. Oasis MAX has the basic structure of Oasis HLB sorbent, modified with a quaternary amine group which has strong anion exchange properties. Benzotriazole compounds should be deprotonated at pHs above 9-10 and no acidic properties are expected for benzothiazoles. Theoretically, the use of Oasis MAX should not result in a specific retention of these compounds, but if the elution is carried out with non-acidified solvents, such as a pure methanol or acetone/methanol mixture (proposed by the authors), clearer extracts with less matrix interferences are obtained compared to Oasis HLB sorbents because of the specific retention of acidic matrix components (mainly humic and fulvic acids) which are not eluted with the proposed procedure. The authors obtained recoveries of over 80% in all the matrices tested and a matrix effect lower than 20% for river water and effluent sewage samples, but for influent sewage samples it increased to around 50% for some compounds (BTR and XTR). Another option for reducing the matrix effect in sewage samples is the method proposed by Herrero *et al.* [26]. As shown in Table 1, the authors determine BTRs, BTs and BSAs in river water and sewage using a combination of two SPE procedures. During the elution step of the Oasis HLB cartridge (where the samples were percolated), a Florisil cartridge is placed at the bottom to retain matrix interferences, and the eluate obtained is analysed by LC-MS/MS. With this procedure, the authors obtained recoveries of over 90% for most of the compounds, and the matrix effect was around 20% for all the matrices tested. Recently, an extraction method using SPME has been proposed by Naccarato *et al.* [37] for the simultaneous determination of some benzotriazoles, benzothiazoles and benzene-sulfonamides, in either environmental aqueous matrices or urine by SPME-GC-MS/MS. In this study, the authors test five different fibers, with polyacrylate being the unique polymeric material tested which was capable of extracting all of the analytes tested. The extraction was in direct immersion mode for 40 min at room temperature and thermally desorbed for GC-MS/MS analysis. The recoveries using SPME were satisfactory for most of the analytes and matrices tested, and matrix effect

was less than 30%. Although the proposed SPME method looks good, the LODs for some compounds may be not enough for their application in some matrices. For example, the authors analysed eleven environmental aqueous samples including tap water, river water and sewage and found none of the selected analytes, when some of them are considered ubiquitous water contaminants.

Another technique used that is based on the same extraction principle of SPME is SBSE, in which a magnetic stirring bar coated with a small amount of sorptive material is used. This technique minimises the use of organic solvents and achieves sensitive and methods because of its higher surface area. Fries [45] uses PA Twister® (polyacrylate) for extracting benzothiazole and 2-methylbenzothiazole in river water and influent sewage samples, and subsequent analysis by thermal desorption-gas chromatography-mass spectrometry obtaining LOD for benzothiazole (256 ng/L) which should be sufficient to determine this compound in some environmental waters. According to the authors, one of the advantages of using SBSE for benzothiazole is the absence of analyte losses, since this compound can be volatilized during sample treatment, resulting in lower recoveries if SPE is used [45]. Moreover, Gilart *et al.* [51] compare different SBSE materials for extracting pharmaceuticals, personal care products and 1-H-benzotriazole from environmental waters by liquid desorption followed by LC-MS/MS analysis. PA Twister®, EG Silicone Twister® (polydimethylsiloxane/ethylene glycol) and PDMS Twister® (polydimethylsiloxane) were tested but the 1-H-benzotriazole was not recovered using any of these materials. The same authors [52] tested a lab-made monolithic coating material for SBSE named poly(PEGMA-co-PETRA), obtaining recoveries of around 10% for 1-H-benzotriazole, which was tentatively quantified in sewage samples.

Notwithstanding the sorptive extraction techniques mentioned above, liquid-liquid extraction (LLE) [46] and dispersive liquid-liquid microextraction (DLLME) [40] have been reported for extracting benzotriazole and benzothiazole compounds. Pervova *et al.* [46] developed a method for extracting 1-H-benzotriazole through its acetylation with acetic anhydride in an aqueous solution in the presence of sodium hydrogen carbonate. The compound formed, *N*-acetylbenzotriazole is then extracted with toluene, with a recovery of about 80%. In addition to this conventional LLE, Pena *et al.* [40] developed a DLLME method for extracting six benzotriazole and five benzothiazole compounds in a wide variety of aqueous samples including include tap, river and industrial waters and influent and effluent sewage. DLLME is based on a ternary component solvent system consisting of a high-density solvent called the extractant, a water-miscible polar solvent called the disperser and the

aqueous sample. Several extractants and dispersers were tested in the aforementioned paper, including ionic liquids. Under optimised conditions, the authors used 9 mL of sample containing 2 g of NaCl and 0.5 mL of methanol as a disperser and 100 µL of tri-n-butylphosphate as an extractant. The recoveries obtained using DLLME were between 67% for 1-H-benzotriazole and 97% for benzothiazole, which were similar to those reported using SPE. DLLME seems to be a promising approach to determine benzotriazole and benzothiazole derivates in environmental waters, saving reagents, time and costs, which may be advantageous for monitoring studies. However, the selection of the suitable solvents is the tricky point of this technique.

Nonetheless, SPE remains the most suitable extraction technique for benzotriazoles benzothiazoles and benzenesulfonamides in aqueous matrices due to its robustness, high recoveries and high preconcentration factors obtained which is required for the low concentration of these compounds in environmental samples.

2.2.2 Solid samples

Environmental solid samples mainly comprise soil, sediments and sewage sludge matrices. By contrast to aqueous samples, there is no predominant extraction technique for solid samples and the number of papers regarding the determination of benzotriazole, benzothiazole and benzenesulfonamide derivates in these matrices is still limited compared to papers regarding aqueous samples. Different extraction techniques for solid samples have so far been applied, as shown in the analytical methods presented in Table 1. The extraction techniques used are pressurised liquid extraction (PLE), ultrasound assisted solvent extraction (USAE) and liquid-solid extraction (LSE), either based on quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction or conventional methods such as shaking. Since most of these extraction techniques use organic solvents, an evaporation step after extraction is mandatory if the sample is analysed using LC- or GC-based methods because of the need to use a more compatible solvent for sample injection or for preconcentration purposes. Moreover, a solid-phase extraction (SPE) is usually performed after extraction as clean-up step.

The sample pre-treatment includes some steps before extraction. The solid samples are usually dehydrated or freeze-dried and crushed and sieved to obtain homogenous particles. The concentrations for the desired compounds are therefore given as the amount of analyte per amount of dry weight (d.w.) sample and the common sample weights are between 0.1 and 1 g (d.w.). Sodium azide is sometimes added to sludge samples after sample collection to suppress microbial activity [23]. As can be seen in Table 1, PLE is one of the most widely used extraction techniques for solid samples. This

extraction technique is faster and consumes less solvent than the other extraction techniques reported, and a semi-automation of the extraction process is possible although specific equipment is required. Liu *et al.* [23] use PLE to extract 4 benzotriazoles from sludge and sediment samples. The extraction was performed with a mixture of methanol/ dichloromethane (1:1) as an extraction solvent at 100ºC for 5 min. The authors mixed the sample with silica to perform an in-cell clean-up. After extraction, evaporation to dryness was carried out to improve the concentration factor and change the solvent to acetone, which is more compatible for GC-MS/MS analysis. The authors obtained recoveries of between 66% and 133% for benzotriazole derivates and LODs of between 0.9 ng/g (d.w.) for BTR and 4.1 ng/g for 5TTR. A similar method, but using methanol/water (1:1) at 80ºC, for the extraction of a wide variety of compounds such as biocides, UV-filters and benzothiazoles in sewage sludge is proposed by Wick *et al.* [25] in an interesting paper in which LC-ESI-MS/MS and LC-APCI-MS/MS methods were also compared. The authors perform an SPE (Oasis HLB) procedure after PLE extraction by diluting the PLE extract to 800 mL to skip the evaporation step prior to SPE. The recoveries obtained were between 46% and 80% for all compounds, except for benzothiazole-2-sulfonic acid, which had a recovery of below 20%. Moreover, Herrero *et al.* [29], proposed pressurised hot water extraction (PHWE) which uses hot water as an extractant in PLE equipment to determine several benzotriazole, benzothiazole and benzenesulfonamide compounds in sewage sludge samples. One of the advantages of PHWE is that it can perform a SPE procedure (e.g., Oasis HLB) directly after extraction. The recoveries using this procedure were between 80% and 101% for most of the compounds, except for ClBTR, BT, and XTR which were between 40% and 60% and around 25% for MeSBT.

Apart from PLE, other extraction techniques have been used for extracting BTRs, BTs and BSAs as shown in Table 1. Asimakopoulos *et al.* [24] developed a USAE method for the extraction of the most common benzotriazole and benzothiazole derivates. The authors used a mixture of methanol/water acidified to pH 3 and ultrasonication for 45 min for extracting the analytes. The supernatant was collected after centrifugation and diluted with ultra-pure water to perform an SPE using a Strata-X cartridge. The recoveries obtained with this procedure were above 90% for all compounds, except for 2-(methylthio)benzothiazole which was around 50%, but the time required for one extraction is much greater than PLE-based methods. The method was applied either to sewage sludge or particulate matter of sewage, and LODs between 0.04 and 12.5 ng/g were obtained, depending on the compound. Zhang *et al.* [53] reported a conventional

liquid-solid extraction with methanol (5 mL) for 30 min, repeated twice, to determine 1-H-benzotriazole, one tolyltriazole isomer and some benzophenone UV filters in sewage sludge and river sediments. After extraction, the supernatant obtained was evaporated and redissolved with ultra-pure water to perform an SPE step using an Oasis HLB cartridge. The recoveries reported by the authors were around 70% for sludge and 80% for sediment samples and the LODs and LOQs were 0.07 and 0.22 ng/g (d.w.), respectively. In addition, Herrero *et al.* [36] developed a method based on QuEChERS for the determination of several benzotriazole, benzothiazole and benzenesulfonamide compounds in sewage sludge by one single extraction. To perform the two-phase partitioning, the authors added 10 mL of ultrapure water to one gram of freeze-dried sludge and then a buffered (citrate) acetonitrile extraction and salting out using anhydrous magnesium sulphate to induce liquid-liquid partitioning was done. An aliquot of the acetonitrile layer was then subjected to a dispersive solid-phase extraction (dSPE) with a zirconium based sorbent (Z-Sep$^+$ from Supelco®) and analysed by LC-HRMS. The recoveries reported were higher than 80% and the LODs were between 1 and 25 ng/g (d.w.). These latter two techniques do not require sophisticated equipment for the extraction of solid samples, which is their major advantage, but in some cases the recoveries are lower and extraction times higher than PLE methods. Nonetheless, QuEChERS has recently been also applied to a broad range of pharmaceutical compounds in sewage sludge with very promising results [54].

3 Occurrence

The occurrence of benzotriazole and benzothiazole derivates in environmental waters has been a focus of study in recent years. However, much less attention has been paid to the occurrence of benzenesulfonamide compounds in the environment. As an example of the interest in benzotriazole compounds, 1-H-benzotriazole and tolyltriazole were included in a pan-European survey on the occurrence of several polar organic persistent pollutants in ground water [55]. In this study, 1-H-benzotriazole was the fourth most commonly detected compound at concentrations higher than 100 ng/L in groundwater, just after bisphenol A. The large number of papers focusing on the occurrence of these compounds in different kinds of environmental water samples is therefore not surprising. Tables 2 and 3 show the occurrence of the three families of compounds covered by this review reported until the beginning of 2014 in different aqueous and solid samples respectively.

As can be seen in Table 2, the most studied compound was 1-H-benzotriazole, which was found between 0.01 and 5.44 μg/L in river water. Concentrations for tolyltiazole

Table 2. Occurrence of BTRs, BTs and BSAs in river water and sewage samples (μg/L).

Compound	Influent sewage	Effluent sewage	River water	Country	Ref.
Benzotriazoles					
BTR	5-7	2-3	-	Australia	[35,42]
	1-44	1-10	<0.05-3	Germany	[25,27,39,58,59]
	0.5-3	0.01-0.5	-	Greece	[19,43]
	0.5-210	0.06-8	0.05-7	Spain	[20,26,34,50,60]
	13-75	11-100	0.06-5	Switzerland	[29,57]
	-	<8	-	The Netherlands	[17]
	-	0.8-4	0.2	UK	[15]
4TTR	2-6	1-2	<0.05-0.3	Germany	[25,27,59]
	<0.06-11	0.04-7	0.03-0.9	Spain	[20,21,34,50]
	-	3-6	0.4	UK	[15]
	-	-	<80-1670	USA	[55]
5TTR	5-8	0.4-0.9	-	Australia	[35,42]
	1-5	0.5-2	<0.05-2	Germany	[25,27,39,59]
	<0.06-5	0.02-17	<2	Spain	[20,21,34,50]
	1-2	-	0.04-2	Spain	[20]
	-	3-6	0.4	UK	[15]
	-	-	<80-2160	USA	[55]
TTR	-	5	0.7	Germany	[58]
	3-16	0.3-6	-	Greece	[19,43]
	0.4-91	0.9	0.2	Spain	[26]
	0.2-6	0.1-4	0.1-625	Switzerland	[29,57]
	-	3	-	The Netherlands	[17]
XTR	0.9-2	0.1-0.2	-	Australia	[35,42]
	0.02	0.01	-	Germany	[27]
	<27	<30	-	Greece	[43]
	<0.01	0.002-0.07	0.002	Spain	[20]
ClBTR	0.6-2	0.08-0.3	-	Australia	[35,42]
	<0.01-14	<0.005	0.002-0.02	Spain	[20,21]
OHBTR	0.1-0.5	<0.2		Greece	[19,43]
	12	-	-	Spain	[21]
Benzothiazoles					
BT	-	2	-	China	[40]
	0.4-1	0.07-12	<0.02	Germany	[23,30,36,39,40]
	0.5-1	<0.05-0.6	-	Greece	[19,43]
	0.2-1	<0.1-3	0.03-0.2	Spain	[20,26,34,50,60]

Table 2. (Cont.).

Compound	Influent sewage	Effluent sewage	River water	Country	Ref.
OHBT	-	1	-	China	[40]
	0.2-0.8	0.1-512	<0.02	Germany	[23,24,30,39,40]
	0.3-0.9	0.09-0.5	-	Greece	[19,43]
	0.1-11	0.005-3	0.006-0.3	Spain	[20,22,26,34,50]
MeSBT	-	0.5	-	China	[40]
	0.2-0.4	0.2-13	0.01	Germany	[23,24,30,40]
	0.2-4	0.04-0.4	-	Greece	[19,43]
	0.1-13	0.06-1	<0.01-13	Spain	[20-22,26,34,50,60]
NH_2BT	-	0.2-0.4	-	Germany	[23]
	<0.02	<0.03	-	Greece	[19,43]
	0.02-9	<0.02	0.004-0.02	Spain	[20,21]
SHBT	-	0.04	-	China	[40]
	0.02-0.2	0.01-747	-	Germany	[23,40]
	0.3-18	0.3	-	Spain	[21,26]
SO_3BT	-	2	-	China	[40]
	1-2	0.4-90	0.07	Germany	[23,24,30,40]
MeBT	-	<0.1	-	Germany	[23]
MeSHBT	0.2	0.1-0.2		Germany	[39]
MorBT	0.01-0.02	0.009-0.02	0.0008	Germany	[30]
TCMTBT	-	<0.2	-	Germany	[23]
Benzenesulfonamides					
BSA	<0.05-1	<0.1-0.6	<0.05-0.5	Germany	[10,28]
	<0.01-0.05	<0.04	0.006-0.01	Spain	[20]
pTSA	<0.05-50	<0.05-11	0.09-1	Germany	[10,28]
	0.1-0.2	0.09-0.4	0.03-0.2	Spain	[20,34,60]
oTSA	<0.05-4	<0.05-3	<0.05-0.8	Germany	[10,28]
	0.04-0.09	<0.080	0.008-0.02	Spain	[20]
MepTSA	<0.01	<0.001-0.005	-	Spain	[20]
EtmTSA	-	-	<0,05	Spain	[34]
EtoTSA	2	0.1	0.08-0.2	Spain	[34]
EtpTSA	0.04-0.8	0.002-0.1	0.002-0.1	Spain	[20,34]

isomers were also reported in several papers but in most of these papers only one isomer was included and detailed data about the performance of the chromatographic method employed to separate them was not reported. In these cases, it is therefore more appropriate to report the concentration as tolyltriazole (mixture of both isomers) instead of 4- or 5-tolyltriazole individually. The individual concentrations of 4- and 5-

tolytriazole have environmental significance because 4-tolyltriazole has been shown to be more resistant to biodegradation than the 5-tolyltriazole isomer [19]. In addition, the removal of these compounds during sewage treatment plants is very limited, as can be observed by the concentrations found in effluent sewage. For example, Asimakopoulos *et al.* [24] found removal efficiencies for benzotriazole compounds from 25% (BTR) to 68% (TTR). These results are comparable to those reported by Reemtsma *et al.* [42] who studied the removal of benzotriazole compounds in the four largest sewage treatment plants in Berlin, and found that the removal efficiencies are highly variable between plants.

Regarding benzothiazole derivates, the most studied compounds were benzothiazole, 2-hydroxybenzothiazole and 2-(methylthio)-benzothiazole, as shown in Table 2. Nonetheless, the occurrence of 2-aminobenzothiazole, 2-mercaptobenzothiazole and benzothiazole-2-sulfonic acid were also reported in some papers and Reemstma [34] and Wick *et al.* [25] found other rare 2-subsituted benzothiazoles in surface water and sewage. As can be seen in Table 2, the occurrence of benzothiazole compounds in river water is less studied than sewage, although some are found at hundreds of ng/L such as 2-(methylthio)-benzothiazole and 2-hydroxybenzothiazole. Their concentration in sewage is in some cases at around μg/L levels for benzothiazole or 2-hydroxybenzothiazole.

As well as the occurrence of these compounds in environmental waters, some studies have also focused on their removal [24,28,47] and degradation [56-59] during sewage treatment either by using conventionally activated sludge or other advanced sewage treatments. Asimakopoulos *et al.* [24] calculated removal for benzothiazole compounds, which ranged between 64% for 2-hydroxybenzothiazole to 91% for benzothiazole. Moreover, Herzog *et al.* [56] and Yuan *et al.* [58] studied different parameters which could improve the sewage removal of benzotriazole compounds by biodegradation using activated sludge. Both studies agree that nitrogen availability for microorganism community in sludge is a crucial factor for the removal of these compounds by biodegradation. In addition, Müller *et al.* [57] studied the degradation products of tolyltriazoles generated by advanced ozonation treatment and the authors found that these compounds are subjected to oxidation of the methyl group to carboxylic acid and the formation of aldehydes and ketones by ring cleavage. Moreover, Mawhinney *et al.* [59] studied the transformation of BTR by ozonation and found the same degradation mechanisms as before.

As mentioned above, benzenesulfonamide compounds are the least studied group of compounds included in this review. To the moment, there were only studies from Spain and Germany, and the concentrations found were up to

μg/L in some cases. *Para*-toluenesulfonamide was the compound found at the highest concentrations in both river and sewage samples, due to its widespread use for different applications such as the synthesis of saccharin, among others. Richter *et al.* [10,35] studied the degradation of benzene-sulfonamide, *para*-toluenesulfonamide and *ortho*-toluenesulfonamide during the watercycle of Berlin. The authors found that the behaviour of these three compounds during sewage treatment was different. While around 90% of *para*-toluenesulfonamide was removed during treatment, the concentration of *ortho*-toluene-sulfonamide increased, decreased or remained equal during treatment and the concentration of benzene-sulfonamide in effluents was increased by 4-6 times compared to influents. Nonetheless, more studies to determine the causes of benzenesulfonamide formation during sewage treatment are necessary.

The occurrence of BTRs, BTs and BSAs in solid samples has been studied to a lesser extent than in aqueous samples. This is probably because due to their poor removal during sewage treatment, they are not expected to be found at high concentrations. However, as shown in Table 3 there are some studies reporting the presence of these compounds at considerable concentrations either in sludge or sediment samples. As with aqueous samples, benzotriazoles are the most studied compounds followed by benzothiazoles. To our knowledge, the occurrence of benzene-sulfonamide compounds in sludge has only been studied by the authors of this review and only toluenesulfonamide (a mixture of *para*- and *ortho*-toluensulfonamide isomers) has been determined at concentrations higher than 80 ng/g [29,36]. Several benzotriazole and benzothiazole compounds have been found in sewage sludge at hundreds of ng/g levels in different studies. For example, Asimakopoulos *et al.* [24] reported concentrations for BTR, TTR, BT, OHBT and MeSBT of above 100 ng/g (d.w.) for most of the compounds in sewage sludge from Greek STPs. Conversely, OHBTR, XTR and NH_2BT were not detected in sludge because these compounds are usually found at low concentrations in sewage. However, Liu *et al.* [49] also found XTR and ClBTR at concentrations higher than 100 ng/g (d.w.) in sludge samples from Australian STPs and Zhang *et al.* [53] found BTR and 5TTR in sewage sludge from Chinese STPs at similar concentrations. Moreover, these latter authors [53] also found the same compounds in river sediment samples from China and the USA, but the concentrations found in the Chinese sediments were ten times as low as the USA sediments.

4 Conclusions

Although the determination of benzotriazoles, benzothiazoles and benzenesulfonamides started more than one decade ago, several studies

Table 3. Occurrence of BTRs, BTs and BSAs in sediment and sewage sludge samples (ng/g (d.w.)).

Compound	Matrix	Concentration	Country	Ref.
Benzotriazoles				
BTR	Sediment	0.4	China	[47]
	Sediment	0.4-33	USA	[47]
	Sludge	<3-219	Australia	[35,42]
	Sludge	17-198	China	[47]
	Sludge	76-412	Greece	[19,43]
	Sludge	<1-27	Spain	[32,46]
	Soil	<3-4	USA	[18]
4TTR	Sludge	<1-82	Spain	[32,46]
	Soil	<2-424	USA	[18]
5TTR	Sediment	2-165	USA	[47]
	Sludge	19-98	Australia	[35,42]
	Sludge	30-104	China	[47]
	Sludge	<1-30	Spain	[32,46]
	Soil	2-168	USA	[18]
TTR	Sludge	72-205	Greece	[19,43]
XTR	Sludge	<10-121	Australia	[35,42]
	Sludge	<22	Greece	[43]
	Sludge	1-11	Spain	[32,46]
ClBTR	Sludge	21-114	Australia	[35,42]
	Sludge	<0.5-7	Spain	[32,46]
Benzothiazoles				
BT	Sludge	265	Germany	[30]
	Sludge	<174	Greece	[19,43]
	Sludge	<26	Spain	[32,46]
OHBT	Sludge	33-312	Greece	[19,43]
	Sludge	<0.5-255	Spain	[32,46]
	Sludge	307	Germany	[30]
MeSBT	Sludge	157	Germany	[30]
	Sludge	23-77	Greece	[43]
	Sludge	<40	Spain	[32]
NH2BT	Sludge	<34	Greece	[43]
	Sludge	<174	Spain	[46]
MorBT	Sludge	5	Germany	[30]
SO_3BT	Sludge	326	Germany	[30]
Benzenesulfonamides				
TSA	Sludge	<84	Spain	[32,46]

are still nowadays focused on developing more sensitive and selective new analytical methods for their determination. Until recently, these different groups of emerging organic contaminants have been determined separately. However, the current trending is to determine them simultaneously in one single analysis as can be seen in the growing number of publications which attempt their simultaneously determination in different environmental samples.

There is a consensus for aqueous samples in which balanced polar/non-polar polymeric SPE sorbents is the preferred option for sample extraction. Nonetheless, for solid matrices different extraction techniques such as PLE or USAE seem to be useful, using relatively polar solvents or mixtures with water as the extractant. Regarding the instrumental analysis techniques used for these compounds, LC-MS/MS seems to be the most powerful technique for their simultaneous determination. Nonetheless, GC-based methods are also used because they are more compatible with miniaturised extraction techniques which have recently been applied for their determination, such as SPME or SBSE. One of the weakest points of LC-based methods is the difficulty in chromatographically resolving the isomeric substances, due to their low retention in LC reversed-phase columns which sometimes does not allow a proper identification of each isomer.

Several benzotriazole, benzothiazole and benzenesulfonamide are found in environmental waters such as surface water and sewage at concentration ranged from few ng/L to hundreds of μg/L, depending on the compound and matrix. In addition, in the studies focused on their removal during sewage treatment it has been observed that benzotriazoles are not effectively removed by conventional treatments. However, higher removal rates have been reported for benzothiazoles. Regarding benzene-sulfonamides, very few papers are focused on their determination and much less in their removal but opposite behaviour has been reported depending on the compound, as for example, benzenesulfonamide increased their concentration after sewage treatment while *para*-toluenesulfonamide is removed. Nonetheless, more studies for benzenesulfonamides are necessary because the little information available about their occurrence, fate and behaviour in the environment is not clear.

To the moment, few studies have been focused on the analysis of environmental solid samples. In these studies, some benzotriazoles, benzothiazoles and benzene-sulfonamides have been found in soil, sediment and sludge at concentrations of hundreds of ng/g. Therefore, more studies seem to be necessary to assess the occurrence of these compounds in solid environmental samples. Moreover, studies especially focused on soil samples located in areas surrounding hot-spots for these compounds such as airports or siderurgy may have

surprising amounts of these chemicals as occurred in some aqueous samples which were highly affected by surrounding areas.

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UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
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CHAPTER 2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
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The main objective of this Doctoral Thesis is the development of analytical methods to determine little studied families of emerging organic contaminants and the evaluation of their presence in environmental samples. The compounds studied in this Thesis are glucocorticoids, polyether ionophores, benzotriazoles, benzothiazoles, benzenesulfonamides and fullerenes.

To achieve this objective both cutting-edge extraction and determination techniques will be assessed. The occurrence of these emerging organic contaminants in river water, sewage and sewage sludge samples will be studied as well as their removal in sewage treatment plants.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As previously discussed in the introduction of this Thesis, most emerging organic contaminants (EOCs) are not effectively removed during conventional treatments at sewage treatment plants (STPs). Thus, several EOCs are still present in the effluent sewage of STPs and reach surface water when these effluents are discharged. Moreover, the reuse of sewage sludge as manure can contribute to the introduction of certain EOCs into different environmental compartments because they are accumulated in sewage sludge during sewage treatment.

EOCs comprise a large number of chemical compounds because new compounds are still being found every day in different environmental matrices and they are likely to be considered as EOCs in the near future. Hence, information about the occurrence, fate and behaviour of many of these new EOCs is not available or is still very limited, both in terms of environmental waters and solids.

As mentioned before, the research of this Thesis focuses on little studied families of EOCs in environmental matrices. As such, the groups of compounds included in are glucocorticoids, polyether ionophores, benzotriazoles, benzothiazoles, benzenesulfonamides and fullerenes. This Thesis has been developed in the Chromatography and Environmental Applications research group at the Universitat Rovira i Virgili, which has extensive experience in the determination of EOCs. This Thesis aims to increase the current knowledge about the presence of the aforementioned families of EOCs in environmental waters and solids by developing analytical methods for their determination in surface water, sewage and sewage sludge samples, for which scarce information is available. The results of this Thesis are related to the projects (I) General Research Directorate of the Ministry of Science and Technology of Spain (CTM2011-28765-C02-01) and (II) NanoNextNL Joint Research Programme of the Dutch Water Utilities (BTO).

This chapter includes the experimental part and results from the different studies that have been carried out through the course of this Thesis. These results have already been published, or are in the process of being published, in international scientific journals, and the following sections are organised accordingly. In these sections, the results are presented in paper format. In each section, a brief introduction is included to establish the context of the research, and the most notable results are also discussed after the papers. The list of the published papers resulting from this Thesis is included in Appendix II.

In the first section, two new methods for the determination of glucocorticoids in environmental waters and sewage sludge are developed. UHPLC-(ESI)MS/MS with triple quadrupole analyser has been used. In both studies, the extraction has been performed using SPE for water sample analysis and PLE for sewage sludge samples. These methods have been applied to the analysis of samples from STPs located in the cities of Tarragona and Reus and the rivers Ebre, Ter and Llobregat in Catalonia.

In the second section, two methods by using LC-(ESI)MS/MS have been developed for determining polyether ionophores in environmental samples. As in the case of glucocorticoids, SPE has been used for the extraction of polyether ionophores from river water samples (Ebre, Ter, Llobregat and Francolí) and sewage samples (STPs in Tarragona and Reus), and PLE has been used for their extraction from sewage sludge samples (STPs in Tarragona, Reus, Blanes, Castell-Platja d'Aro and Palamós). Moreover, this section includes a comparison of tandem mass spectrometry and high resolution mass spectrometry for the simultaneous determination of glucocorticoids and polyether ionophores using UHPLC-(ESI)QqQ-MS/MS and UHPLC-(HESI)Orbitrap-HRMS which was performed in collaboration with the Mass Spectrometry Laboratory/Organic Pollutants Department at the Institut de Diagnosi Ambiental i Estudis de l'Aigua/Consell Superior d'Investigacions Científiques (IDAEA-CSIC) in Barcelona.

The third section covers the development of three analytical methods for the simultaneous determination of benzotriazoles, benzothiazoles and benzenesulfonamides in either environmental waters or sewage sludge. For environmental waters, a method based on tandem SPE followed by LC-(ESI)MS/MS has been developed, while, for sewage sludge, two extraction methods based on QuEChERS and PWHE both followed by LC-(HESI)Orbitrap-HRMS have been developed. The SPE/LC-(ESI)MS/MS method has been applied to analyse water samples from four Catalan Rivers (Ebre, Ter and Llobregat and Tordera) and six STPs located in Tarragona, Reus, Vila-Seca/Salou, Blanes, Castell-Platja d'Aro and Palamós, of which the last four have tertiary treatments. Both QuEChERS and PHWE extraction have been used for analysing sewage sludge samples from the STPs located in Tarragona, Reus, Blanes, Castell-Platja d'Aro and Palamós. The samples from Girona region were supplied by the Group of Analytical and Environmental Chemistry of the Universitat de Girona which participates in the project CTM2011-28765-C02.

The last section focuses on the development of two methods using a recently upgraded separation technique (AF4) for the size determination of aqueous fullerene aggregates. This study was conducted in collaboration with the KWR Watercycle Research Institute in Nieuwegein (the Netherlands), during a European placement conducted during the course of this Thesis. The studies included in this section are on hyphenated AF4 with high resolution mass spectrometry by using an APPI interface to improve the sensitivity and selectivity of previous methods based on UV-Vis or MALS detection, which are not suitable for the expected environmental concentrations of fullerenes. Moreover, fractionations of aqueous fullerenes aggregates have been further optimised and an external size calibration has been proposed for their size determination when AF4-(APPI)LTQ-Orbitrap-HRMS is used. The method has been applied for the analysis of river water and sewage samples from the Netherlands.

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3.1. Determination of glucocorticoids in environmental samples

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This section focuses on the determination of glucocorticoid compounds in river water and sewage samples using SPE/UHPLC-MS/MS in the first study, and on the determination of the same compounds in sewage sludge using PLE/SPE/UHPLC-MS/MS in the second study. As mentioned in previous sections, information about the presence of glucocorticoids in environmental samples is still very limited [1-3] and, therefore, the main reason for choosing these compounds was to increase the current knowledge about their occurrence in the environment because of the environmental risk associated to the possible development of bacterial resistance to these drugs and their oestrogenic effect on humans and wildlife [4].

To date, different analytical methods have been developed to determine glucocorticoids in environmental matrices but most of them are based on multiresidue methods which only include one or two glucocorticoids [5] or in which glucocorticoids are determined together with other steroidal compounds [3]. Therefore, specific analytical methods for the determination of glucocorticoids in environmental samples are scarce. Due to the existence of isomeric compounds, such as betamethasone and dexamethasone, for which their simultaneous determination is usually skipped, the first study presented here has a more in-depth focus on the chromatographic separation and mass spectrometric optimisation of glucocorticoids, including these isomers. In this study, a UHPLC column was tested to enhance the separation of these compounds due to the higher efficiency provided by this kind of columns with sub-2 μm particle diameter, as well as the short analysis times obtained. Moreover, this study includes the test of three different sorbents for SPE with different structures and chemical properties (Oasis HLB, Isolute ENV+ and Bond Elut Plexa) to find the best option for their extraction from aqueous matrices.

The second study regards the optimisation of PLE for extracting glucocorticoids from sewage sludge because, to date, only a couple of papers have focused on their determination in this kind of sample using USAE [2,3], while PLE has not been used before for extracting these compounds from sewage sludge. In the study presented, some clean-up strategies have been tested, such on-cell PLE clean-up and SPE clean-up, to reduce the matrix effect that occurs when these samples are analysed by UHPLC-(ESI)MS/MS and then, to improve LODs.

The proposed methods have been applied to analyse river water samples from the rivers Ter, Llobregat and Ebre and, influent sewage, effluent sewage and sewage sludge samples from two STPs located in Tarragona and Reus, because data

regarding the presence of these compounds had never been reported for these STPs.

The results of these studies have been published in the *Journal of Chromatography A 1224 (2012) 19-26* and *Talanta 103 (2013) 186-193*.

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3.1.1. Determination of glucocorticoids in sewage and river waters by ultra-high performance liquid chromatography-tandem mass spectrometry

UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
Pol Herrero Gil
Dipòsit Legal: T 1619-2015

DETERMINATION OF GLUCOCORTICOIDS IN SEWAGE AND RIVER WATERS BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

In this paper we present a method based on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) combined with a solid-phase extraction to determine nine glucocorticoids in river waters and sewage. In addition, we attempt the chromatographic separation of two glucocorticoid epimers (betamethasone and dexamethasone) which until now had not been determined simultaneously in environmental matrices. For SPE, we have tested three commercial polymeric polar/non-polar balanced sorbents. The recoveries were close to or above 90% in rivers and sewage influents and effluents. The repeatability expressed as relative standard deviation (%RSD, n=3, 10 ng/L) was less than 8% in all cases. The method obtains LODs for glucocorticoids at low ng/L levels in aqueous environmental matrices (0.5-20 ng/L depending on the matrix and the analyte). The method was applied to determine these compounds in three Catalan rivers (Ebre, Ter and LLobregat) and two sewage treatment plants in the Tarragona area. Cortisone, cortisol, prednisone and prednisolone were frequently determined in influent sewage samples between 21-285 ng/L. Moreover, the two epimers were successfully determined below LOQs in some influent sewage samples.

***Keywords**: Glucocorticoids; Ultra-high performance liquid chromatography; Tandem mass spectrometry; Solid-phase extraction; Sewage; River water.*

1 Introduction

Pharmaceutical compounds are included in the group of so-called emerging organic contaminants and are frequently used for both human and veterinary use [1,2]. Synthetic glucocorticoids form an important group of these drugs and have a wide range of therapeutic applications, such as in the treatment of asthma or other inflammatory diseases. However, they also have a significant effect as growth promoters [3,4]. Due to bad farming practices around the world they were banned for use as growth promoters in the EU in 1990.
Extensive use of these compounds to treat humans and animals can lead to the transfer of these pollutants to the environment by different ways. Glucocorticoids are poorly absorbed by the organism, which means that around to 50-90% of these drugs are quickly excreted in urine and faeces. Human residues are placed directly into the urban sewer system and are carried to sewage treatment plants. Pharmaceutical residues excreted by animals can be directly filtered into the soil, or if it is used as a fertilizer, it can be scattered over a wide expanse of land. These compounds also get into the environment because they are very frequently administered with fodder (or administered directly into the water in the case of fish farms) and are therefore once more filtered directly into soil [5]. However, the amount introduced into the environment is probably small, and we have no evidence of veterinary drugs persisting in the environment [6]. Nevertheless, a continuous drip feed of these compounds over time may result a relatively high concentration or long term chronic exposure if they are not removed by sewage treatment plants. Glucocorticoids and other classes of steroid hormones are potentially endocrine disrupting chemicals and therefore, detection and identification of these compounds in environmental matrices can help to ensure human health, uncover bad farming practices, prevent hormonal disorders and other diseases in aquatic species, and determine the efficiency of sewage treatment plants in eliminating them.
Several studies have shown that current treatments used in sewage treatment plants (STPs) are not fully effective in eliminating these contaminants [7], which in turn may lead to environmental problems and a risk to human health.
Numerous studies have recently been published regarding the determination of these compounds in biological matrices [8-14] in which maximum residue levels (MRLs) have been established (UE nº 37/2010) [15]. Unfortunately, there are few studies on environmental matrices [1,5,16-23]; however, those that have been carried out confirm the presence of these drugs in the environment. Most studies have been carried out in China, and all of these have found residues of glucocorticoids in sewage influents and, to a lesser extent, effluent sewage [5,17] and sewage sludge [22]. Studies in Japan [24] and France [19] also confirm the presence of these compounds in sewage.

However, studies in river waters [17,25] show no significant presence of these drugs. Only studies in river water in China report a systematic occurrence at levels below ng/L [5,23]. A study in hospital wastewater in the Netherlands [21] reports quite high values for glucocorticoid residues.

Chromatographic methods are the most widely used to determine glucocorticoids in environmental waters. Liquid chromatography [24] and more recently ultra-high performance liquid chromatography [5], gas chromatography [26] and micellar electrokinetic chromatogramphy [27] have been used to determine these compounds in a wide range of matrices. Liquid chromatographic techniques are the most suitable because glucocorticoids are non-volatile compounds which do not have ionisable groups. Consequently they cannot be directly analyzed using conventional GC or CE techniques, which means that it is necessary to apply complementary strategies such as derivatisation in gas chromatography or micelles in capillary electrophoresis. Tandem mass spectrometry [5], fluorescence [28] and UV-Vis [29] are the most commonly used detection techniques in LC. LC-MS/MS with ESI or APCI interface are the preferred techniques due to their high sensitivity and selectivity [4]. Often, a solid-phase extraction pretreatment is necessary to determine glucocorticoids in environmental aqueous matrices because they are present at very low concentrations (low ng/L values). Several papers indicate that polar/non-polar balanced polymeric sorbents, such as Oasis HLB or Strata-X, are the most suitable for this purpose [25].

The complete chromatographic separation of glucocorticoids is not easy because they all have a very similar chemical structure. Betamethasone and dexamethasone are two epimers permitted under European legislation and they are used as anti-inflammatory and immunosuppressant. They do not possess selective fragmentation when subjected to tandem mass spectrometry and therefore it is necessary to separate them completely. However, chromatogramphic separation of epimers is usually omitted from the literature and, therefore, one of our priorities is the chromatographic discrimination between these two epimers and then evaluate their occurrence and fate in rivers and STPs, while the others common glucocorticoids such as cortisol, cortisone, prednisone and prednisolone besides others (methylprednisolone, flumethasone or triamcinolone acetonide) are also evaluated in the present study.

This paper focuses on the development of an analytical method based on an ultra-high performance liquid chromatography-(electrospray)-tandem mass spectrometry (UHPLC-(ESI)-MS/MS) with a previous SPE preconcentration step. The method is designed to simultaneously determine nine glucocorticoids used for both human and veterinary purposes in sewage and river water. The mass

spectrometry fragmentation pathways of these compounds have also been studied. The method has been applied to water samples from two different sewage treatment plants and three Catalan rivers.

2 Experimental

2.1 Reagents and standards

The standards used were betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, dichlorisone acetate, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide. All were purchased from Sigma-Aldrich (St. Louis, USA). The glucocorticoids' chemical structure is presented in Figure 1. Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and storing it at -20 ºC. Fresh stock solutions of 100 mg/L in methanol were prepared every month and stored at 4 ºC. Working solutions were prepared daily by diluting these solutions with water/acetonitrile (4:1).
Ultrapure water was obtained using an ultrapure water purification system from Veolia waters (Sant Cugat del Vallés, Spain). Acetonitrile (ACN), methanol (MeOH) and ethyl acetate (EtOAc) were of HPLC grade from SDS (Peypin, France) and nitrogen gas was from Carburos Metálicos (Tarragona, Spain). Formic acid was purchased from Merck (Darmstadt, Germany) and used to adjust the pH of the mobile phase and the samples.

2.2 Sample preparation

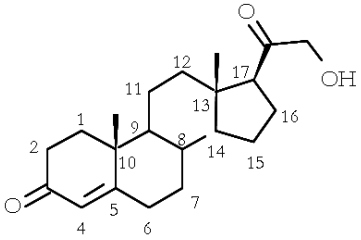
The river water samples were collected from three Catalan rivers (Ebre, Ter and Llobregat) and the sewage samples were collected from the influent and effluent of two urban sewage treatment plants (STPs) located in the area of Tarragona (STP1 and STP2), population is around to 140,000 inhab. for STP1 and 107,000 inhab. for STP2. The STPs receive urban sewages and some industrial discharges and use activated sludge biological treatment. The biological oxygen demand (BOD_5) for influent water is about 400 mg/L at both STPs and the average flow rate is 30,000 m^3/day for STP1 and 16,000 m^3/day for STP2. All samples were collected by using pre-cleaned amber glass bottles and filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborough, UK) and a 0.45 μm nylon filter (Whatman, Maidstone, UK). The samples were then acidified to pH 3 with formic acid to prevent microbial growth and stored at 4 ºC until analysis. Prior to analysis, the samples were allowed to reach room temperature.

2.3 UHPLC-MS/MS conditions

The chromatographic instrument was an Agilent 1200 series (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). It was equipped with an automatic injector, a degasser, a binary pump, and a

column oven. The chromatographic column was a Zorbax Eclipse XDB-C_{18} (50 x 4.6 mm, 1.8 µm) (Agilent Technologies).

A ternary mobile phase with a gradient elution was used. Solvent A was water/acetonitrile (78:22 v/v) with formic acid (0.1%) and solvent B was methanol/acetonitrile (78:22 v/v) with formic acid (0.1%). The gradient was started at 0.8% of B, then increased to 5% in 5 min, 15% in 6.5 min, 50% in 0.5 min, kept constant for 1.5 min, increased to 99.9% in 0.5 min, kept constant for 0.5 min, and finally returned to 0.8% B in 0.5 min. All the compounds eluted within 13.5 min. The oven temperature was kept at 50 ºC to reduce the backpressure and to decrease the retention factor of analytes. The flow rate was 1 mL/min and the injection volume was 50 µL.



Compound		1-2	6	9	11	16	17	16-17
Betamethasone	BMS	=		F	OH	CH_3	OH	
Cortisol	HCOR				OH		OH	
Cortisone	COR				=O		OH	
Dexamethasone	DMS	=		F	OH	CH_3	OH	
Flumethasone	FMS	=	F	F	OH	CH_3	OH	
Methylprednisolone	MPNL	=	CH_3		OH		OH	
Prednisolone	PNL	=			OH		OH	
Prednisone	PNS	=			=O		OH	
Triamcinolone acetonide	TACA	=		F	OH			$OC(CH_3)_2O$

Fig. 1. Chemical structure of glucocorticoids.

A flow injection of a standard solution of each compound was used to find the optimum conditions for each compound in the ESI source. The average conditions selected for the optimum performance of the ESI in the negative mode were: nebulizer pressure 40 psi, drying gas (N_2) flow rate 12 L/min, drying gas temperature 350 °C, and capillary voltage 2000 V. Cone voltage and collision energies were optimized for each compound to obtain three MRM transitions, one for the quantification and two more

for the confirmation of analytes. These are described in Table 1. The ratios between both transitions were used for confirmation purposes. Three time windows were used: 4-8 min (prednisone, prednisolone, hydrocortisone and cortisone), 8-12.5 min (methylprednisolone, beta-methasone, flumethasone and dexamethasone) and 12.5-14 min (triamcinolone acetonide).

2.4 Solid-Phase Extraction

For the SPE procedure we tested a 200 mg Bond Elut Plexa (Varian, Agilent Technologies), a 500 mg Oasis HLB (Waters, Wexford, Ireland) or a 500 mg Isolute ENV+ (IST, Hengoed, UK) connected to a manifold (Teknokroma, San Cugat del Vallés, Spain) with a pump as a vacuum source.

The SPE cartridge used was Bond Elut Plexa (200 mg). It was preconditioned with 5 mL of MeOH followed by 5 mL of water. Volumes for STP influent and effluent water samples were 100 and 250 mL respectively, and 500 mL for river water samples. This sample volume was loaded into the cartridge at a flow-rate of 10–15 mL/min. Before the elution step, the sorbent was dried under vacuum. The retained analytes were eluted from the cartridge with 10 mL of MeOH. The eluate was concentrated under a flow of nitrogen gas to dryness and the residue was redissolved in 1 mL of water/acetonitrile (4:1) for river water and 3 mL of water/acetonitrile (4:1) for influent and effluent sewage.

3 Results and discussion

3.1 UHPLC-MS/MS optimisation

Previous studies have reported that glucocorticoids form different precursor ions depending on the mobile phase and ionisation mode used [5,10]. In the present study, we have studied the formation of precursor ions and their daughter ions under the aforementioned chromatographic conditions. Because glucocorticoids do have not acid-base properties in the pH range, when a C_{18} reverse phase column is used, we chose formic acid at a concentration of 0.1% (v/v), in accordance with the literature, to maximize the ionisation of analyte in the electrospray interface ($[M+H]^+$ in positive mode or $[M+For]^-$ in negative mode). Positive ionisation mode not only shows the $[M+H]^+$ ion, but also the intense formation of a sodium adduct $[M+Na]^+$ and the low intensity formation of a potassium adduct $[M+K]^+$. This results in a decrease in the formation of $[M+H]^+$ ion due to competition from the adduct ionisation pathways. Thus, we attempted maximize the sodium adduct formation by adding a small amount of sodium acetate (100 μM) as the mobile phase additive. This amount had to be small to prevent ion suppression caused by a high concentration of ions species in the electrospray interface. However, we needed to be able to ensure the optimal formation of the sodium adduct and its reproducibility, especially in environmental matrices.

Unfortunately, when we studied the fragmentation of these precursor ions at low collision energies, no fragmentation of the sodium adduct was observed. When these energies were increased, the m/z signal of the adduct ion disappeared as a neutral product and m/z signals were not detected. However, the precursor $[M+H]^+$ produced many fragments at low collision energies, resulting in less intense MRM transitions. Of particular note is the presence of other precursors such as $[M+H-H_2O]^+$ or $[M+H-HF]^+$, which also reduce the positive ionisation mode's suitability for these compounds because they lead to the formation of multiple precursors.

When we worked in the negative ionisation mode, the most abundantly formed precursor ion was $[M+For]^-$ [30], although chloride adducts $[M+Cl]^-$ also appeared. These adducts are less abundant than those generated by sodium in the positive ionisation mode. The precursor ion $[M+For]^-$ generates the highly abundant daughter ion $[M-H-CH_2O]^-$ for the quantification MRM transition, and other fragments for the confirmation MRM transitions. These fragmentation pathways were present in all the compounds studied except triamcinolone acetonide, whose most abundant daughter ion was $[M-H-HF]^-$. As Figure 1 shows, triamcinolone acetonide has a slightly different structure from the other glucocorticoids that have been studied.

The acetonide group $(-OC(CH_2)_2O-)$ creates a heterocyclic ring between the carbons C16-C17 instead of the hydroxyl group in the C17 present in the rest of glucocorticoids. The ketone (C18) and hydroxyl groups (C17 and C19) are strongly related to the formation of adduct with formate (see Figure 2).

Therefore the presence of the acetonide group between the carbons C16-C17 eliminates the interaction by hydrogen bond with the hydroxyl group at C17.

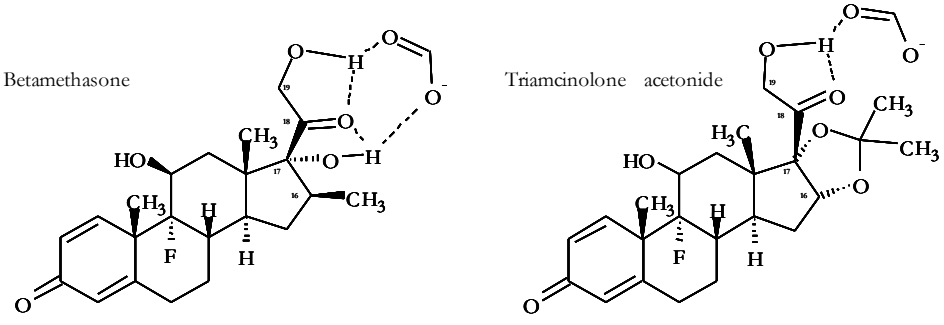


Fig. 2. Formate adduct formation for betamethasone and triamcinolone acetonide.

Table 1. LC-MS/MS acquisition parameters in MRM mode.

Compound	t_R (min)	C. V. (V)	Precursor ion (m/z)	Quantifier ion* (m/z)	Qualifier ion 1* (m/z)	Qualifier ion 2* (m/z)
PNS	4.9	100	403 $[M+For]^-$	327 (10) $[M\text{-}H\text{-}CH_2O]^-$	299 (15) $[M\text{-}H\text{-}CH_2O\text{-}CO]^-$	285 (30) $[M\text{-}H\text{-}CH_2O\text{-}CH_2CO]^-$
PNL	5.0	100	405 $[M+For]^-$	329 (10) $[M\text{-}H\text{-}CH_2O]^-$	280 (30) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}{\cdot}CH_3]^-$	295 (30) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$
HCOR	5.4	100	407 $[M+For]^-$	331 (10) $[M\text{-}H\text{-}CH_2O]^-$	297 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$	282 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}{\cdot}CH_3]^-$
COR	5.5	100	405 $[M+For]^-$	329 (10) $[M\text{-}H\text{-}CH_2O]^-$	301 (15) $[M\text{-}H\text{-}CH_2O\text{-}CO]^-$	311 (30) $[M\text{-}H\text{-}CH_2O\text{-}H_2O]^-$
MPNL	9.9	110	419 $[M+For]^-$	343 (10) $[M\text{-}H\text{-}CH_2O]^-$	294 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}{\cdot}CH_3]^-$	309 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$
BMS	10.4	110	437 $[M+For]^-$	361 (15) $[M\text{-}H\text{-}CH_2O]^-$	307 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}HF\text{-}H_2O]^-$	292 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}HF\text{-}H_2O\text{-}{\cdot}CH_3]^-$
FMS	10.5	110	455 $[M+For]^-$	379 (15) $[M\text{-}H\text{-}CH_2O]^-$	305 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}2HF\text{-}H_2O]^-$	325 (30) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}HF\text{-}H_2O]^-$
DMS	11.1	110	437 $[M+For]^-$	361 (10) $[M\text{-}H\text{-}CH_2O]^-$	307 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}HF\text{-}H_2O]^-$	292 (35) $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}HF\text{-}H_2O\text{-}{\cdot}CH_3]^-$
TACA	13.1	110	479 $[M+For]^-$	413 (15) $[M\text{-}H\text{-}HF]^-$	337 (20) $[M\text{-}H\text{-}C_2H_6CO\text{-}HF\text{-}H_2O]^-$	375 (10) $[M\text{-}H\text{-}C_2H_6CO]^-$

*Collision energies are in brackets (eV).
C.V. is cone voltage.

Thus, it seems that the elimination of the (CH_2O) group is intrinsically linked to the elimination of the formate. Some doubt may arise as to whether the daughter ion observed is $[M\text{-}H\text{-}CH_2O]^-$ or $[M+For\text{-}C_2H_2O_2\text{-}H_2O]^-$ due to the fact that both have the same m/z ratio. However, the literature always reports that [M-H-CH_2O]- is the daughter ion and studies with acetic acid have obtained the same daughters ions from different parents ions $[M+AcO]^-$. This indicates that the acid is necessarily eliminated and that the daughter ion generated is the $[M\text{-}H\text{-}CH_2O]^-$. All of this confirms the hypothesis that the formate (or acetate) and the (CH_2O) group are jointly eliminated because this neutral loss is not observed in triamcinolone acetonide, where the interaction between formate and analyte is much more labile than in other glucocorticoids.

The parameters that affect the performance of the ESI interface in both modes were individually optimized for each compound. The variables optimized and the intervals tested were: nebulizer pressure (20 to 60 psi), drying gas temperature (150 to 350 ºC), drying gas flow (5 to 13 L/min), capillary voltage (1500 to 6000 V), and cone voltage (0 to 200 V). The values that provided the best response were selected as the optimum values for each compound and are described in Section 2.4.

The mobile phase pH and temperature of the column have not been studied experimentally because these analytes do not show acid-base

properties and therefore the pH will not have a significant effect on their chromatographic separation. The temperature did not affect the selectivity of these compounds because they have extremely similar structures. Only, an increase in column temperature decreases the retention factor of the analytes alike. Given that we were using an UHPLC column (1.8 μm), we chose a temperature of 50 °C to reduce the system backpressure. We also reduced the analysis time and did not submit the column to stress, in accordance with manufacturer's specifications. We studied between 0.1% and 0.3% (v/v) of formic acid to determine whether there had been any improvement in the ionisation of the analytes.

Using a ternary mobile phase provides many benefits in the separation of glucocorticoids. The epimers dexamethasone and betamethasone cannot be separated with methanol alone, but they can be separated with acetonitrile, as reported in the literature [31]. This has been confirmed experimentally, but unfortunately the use of acetonitrile does not provide sufficient selectivity to separate the set of more polar compounds (prednisone, prednisolone, cortisol and cortisone) or to differentiate between the flumethasone and betamethasone compounds. However, methanol does separate these compounds. We have used a quaternary pump to optimize the gradient elution and we are working with three solvents (water, methanol and acetonitrile) to find a composition of acetonitrile, which that remains constant throughout the separation and thus allows the use of a binary pump in the final method. Separation of glucocorticoids shows a characteristic feature. All compounds show different ranges of retention times depending on similar slight

Table 2. Recoveries and RSDs (%, n=3) for tested elution solvents.

Compound	EtOAc		MeOH	
PNS	50	(3)	95.6	(0.9)
PNL	31	(10)	96.3	(0.7)
HCOR	32	(13)	92.5	(0.4)
COR	58	(8)	96	(1)
MPNL	32	(22)	101.1	(0.5)
BMS	25	(21)	102	(1)
FMS	28	(16)	100.6	(0.5)
DMS	27	(19)	102.8	(0.5)
TACA	82	(5)	110	(1)
DCSA	101	(5)	94	(3)

Sorbent: Oasis HLB 500 mg.
Load volume: 100 mL of ultrapure water spiked at 50 μg/L.
Elution volume: 5 mL.

variations in their structure. Thus, the first group which contains prednisone, prednisolone, hydrocortisone and cortisone compounds appears between 4.9 and 5.5 min. This set of compounds has a common structure (similar to cortisol) because all glucocorticoids are a derivates of cortisol. Methylprednisolone, betamethasone, flumethasone and dexamethasone elute between 9.9 and 11.1 min. These compounds have a methyl group linked to the carbons C6 or C9. Finally, triamcinolone acetonide shows a retention time of 13.1 min and this high retention factor is due to the presence of the acetonide group between the C16 and C17 carbons. This compound could be run faster, but if the percentage of organic phase increases more quickly also increases the ion suppression in this range. Because of this, it is necessary elute triamcinolone acetonide slowly in order to separate it from interfering substances that would not allow its determination.

3.2 SPE procedure

Since we expect to find the compounds under study at very low concentrations in the environment, it is necessary to apply a SPE procedure before chromatographic analysis. Different papers and our prior knowledge led us to choose polymeric sorbents as the most suitable for initiating the study of the SPE procedure [5,10,32,33]. Polar/non-polar balanced polymeric sorbents have good affinity with slightly polar and non-polar compounds, such are the majority of drugs. In this study we compared three commercial polymeric sorbents: Oasis HLB (vinylpirrolidone-divinylbenzene) and Bond Elut Plexa (Hydroxylated surface and polystyrene-divinylbenzene core) have a macroporous structure, and Isolute ENV+ (Hydroxylated polystyrene-divinylbenzene) has a hypercrosslinked structure. We started by testing Oasis HLB because it is the most commonly used. The pH was not expected to have any effect on the extraction of the analytes because they do not have any ionisable group, and this was corroborated by the results obtained in the two different pHs studied (3 and 7). However, the SPE procedure was done at pH 3 because the samples had been acidified to this pH with formic acid to prevent microbial growth. The elution of the compounds was tested with two different solvents (MeOH and EtOAc) and different volumes (2-10 mL). The results in Table 2 show that MeOH is the best solvent for extracting glucocorticoids because ethyl acetate is unable to efficiently extract the compounds and only recoveries from 25 to 50% were obtained by the majority of studied glucocorticoids. Furthermore, 10 mL of methanol compared with smaller volumes slightly improved the glucocorticoid recoveries. Also, loading volumes of 500 and 1000 mL with ultrapure water were tested, but no breakthrough was found. Therefore, we decided to use a

Table 3. Recoveries and RSDs (%, n=3) for tested sorbents.

Compound	Oasis HLB		Bond Elut Plexa		Isolute ENV+	
PNS	93.4	(0.9)	98.8	(0.8)	77	(8)
PNL	94	(1)	97.6	(0.6)	95	(2)
HCOR	96	(0.7)	98.3	(0.3)	90	(6)
COR	97.1	(0.4)	97.3	(0.6)	70	(9)
MPNL	98	(1)	98.9	(0.1)	99.6	(0.4)
BMS	94.5	(0.8)	99.1	(0.7)	97.5	(0.3)
FMS	93	(2)	98.2	(0.9)	97.8	(0.7)
DMS	98.3	(1)	97.2	(0.5)	100.0	(0.3)
TACA	96	(4)	100.0	(0.1)	91	(2)
DCSA	99.7	(0.1)	100.1	(0.6)	16.9	(0.1)

Elution solvent: MeOH (10 mL).
Load volume: 1000 mL of ultrapure water spiked at 50 μg/L.

volume of 1000 mL for the subsequent tests. The other polymeric sorbents (Bond Elut Plexa and Isolute ENV+) were tested under the conditions that had been previously optimized by the Oasis HLB sorbent in order to gauge their suitability. The results are shown in Table 3 which shows that both Bond Elut Plexa and Oasis HLB are the most suitable sorbents and have very similar recoveries (>90%) and repeatability (<1%) for glucocorticoids. Isolute ENV+ does not offer a good recovery (only 17%) for the most non-polar compound (dichlorisone acetate) and the recoveries obtained for the other glucocorticoids are lower (>70%) than the recoveries obtained with other sorbents under study. Therefore, both Bond Elut Plexa and Oasis HLB are suitable for carrying out an SPE procedure for glucocorticoids. Of these two sorbents, we chose the Bond Elut Plexa sorbent because less research has been conducted on it than on Oasis HLB. This meant that by studying it we could increase the range of possible SPE sorbents.

In addition, we also evaluated the evaporation to dryness of the extract and its redisolution in 1 mL of mobile phase to achieve a higher preconcentration factor and found no significant analyte losses.

Conversely, when working with environmental samples, it was likely that we would need to reduce the load volume sample depending on its origin (river water, influent or effluent sewage) because of the complexity of these matrices. We evaluated the load volume for different kinds of environmental samples and the results led us to choose a sample volume of 500 mL for river water, 250 mL for STP effluent and 100 mL for STP influent.

In first instance, the matrix effect was studied to determine whether we could use an external standard calibration curve to quantify the analytes. The matrix effect was

evaluated in the three matrices (river water, STP influent and STP effluent) and was calculated by comparing the signal response obtained when spiking a sample blank after extraction with the signal response obtained from a standard solution at the same concentration. We were then able to see that all matrices cause ion suppression in all the analytes. In river water ion suppression was around 20%, but in sewage samples ion suppression increased to 65% for some analytes.

Table 4. Validation values for the SPE-LC-MS/MS method.

Compound	LOD (ng/L)	Linear range (ng/L)	Recovery[a] (%, n=3)	Repeatability[a] (%RSD, n=3)
Betamethasone	0.5*	1.5-100	94	2
	3.0**	10-600	92	4
	7.5***	20-1500	95	2
Cortisol (Hydrocortisone)	0.5	1.5-100	97	3
	3	10-600	96	3
	7.5	20-1500	97	3
Cortisone	0.5	1.5-100	91	3
	3	10-600	93	6
	7.5	20-1500	88	2
Dexamethasone	0.5	1.5-100	92	1
	3	10-600	90	3
	7.5	20-1500	91	6
Flumethasone	0.5	1.5-100	87	1
	3	10-600	94	5
	7.5	20-1500	91	7
Methylprednisolone	0.5	1.5-100	94	2
	3	10-600	93	7
	7.5	20-1500	93	5
Prednisolone	0.5	1.5-100	99	2
	3	10-600	90	8
	7.5	20-1500	96	3
Prednisone	0.5	1.5-100	82	4
	3	10-600	98	2
	7.5	20-1500	96	3
Triamcinolone acetonide	1.5	5-100	96	2
	9	20-600	90	5
	20	50-1500	97	4

* River water, ** Effluent sewage and *** Influent sewage.

[a] River water, effluent and influent sewage spiked at 10, 20 and 30 ng/L, respectively.

Table 5. Qualifier ion ratios in spiked samples and concentrations (ng/L) of glucocorticoids in STP1 sewage.

Compound	Qualif. 1 ratio (%)	Qualif. 2 ratio (%)	October 2010		December 2010		February 2011		April 2011	
			Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
PNS	20	9	<20	n.d	<20	<10	<20	<10	21 (24, 11)	n.d
PNL	15	13	25 (17, 12)	n.d	<20	n.d	<20	n.d	24 (16, 13)	n.d
HCOR	15	16	190 (17, 19)	<10	n.d	n.d	n.d	n.d	136 (18, 16)	<10
COR	21	8	135 (24, 9)	n.d	n.d	n.d	n.d	n.d	122 (23, 7)	n.d
MPNL	20	21	<20	n.d	<20	n.d	n.d	n.d	n.d	n.d
BMS	24	18	<20	<10	<20	n.d	n.d	n.d	<20	n.d
DMS	19	14	<20	<10	<20	n.d	n.d	n.d	<20	n.d
TACA	98	78	<50	n.d	n.d	n.d	n.d	<20	n.d	<20

* Qualifier ion ratios (%) in samples are in brackets.

We would have used a deuterated surrogate as the internal standard to correct the error caused by ion suppression, but its low commercial availability and high price led us to reject this possibility. Given that the aim of this paper is to study the presence of glucocorticoids in different kinds of waters and to evaluate their elimination in sewage treatment plants, we looked for a compound with similar structural and chemical properties. As result, we chose dichlorisone acetate as the internal standard in agreement with the literature [10]. Unfortunately, the chromatographic retention of this compound is much higher than the other glucocorticoids under study, which means that they are not suitable for use as a matrix effect corrector. In addition, their fragmentation pathways and signal responses are too different from those of the target compounds. All this resulted in the decision not to use the internal standard in our study, a decision supported by the high reproducibility obtained in the SPE procedure. We then used a matrix matched calibration curve to correct the ion suppression and recovery of the analytes.

Recovery of the analytes in environmental samples was evaluated by comparing them with a blank sample spiked before and after the SPE procedure at the same final concentration. The evaporated extracts were redissolved with a volume of 1 mL of mobile phase. In addition, a blank sample was evaluated in order to subtract the

possible signal generated. The experimental results show that recoveries for all analytes exceeded 95% in all kinds of water samples (river and sewage).

Table 6. Concentrations (ng/L) of glucocorticoids in STP2 sewages.

Compound	February 2011		April 2011	
	Influent	Effluent	Influent	Effluent
PNS	45 (22, 11)	<10	23 (24, 11)	n.d
PNL	33 (17, 10)	n.d	25 (16, 13)	n.d
HCOR	270 (17, 20)	<10	136 (16, 18)	<10
COR	285 (23, 9)	n.d	122 (23, 7)	n.d
MPNL	<20	n.d	n.d	n.d
BMS	<20	<10	<20	n.d
DMS	<20	<10	<20	n.d
TACA	n.d	n.d	n.d	<20

* Qualifier ion ratios (%) in samples are in brackets.
* Values in spiked samples are in Table 5.

We then decided to evaluate how to reduce the matrix effect. As has been discussed, the load sample volumes used do not affect the correct recovery of the analytes and only cause a signal loss due to ion suppression. Thus, an easy and quick solution would be the redisolution volume of the SPE extracts. To do this, we looked for a suitable dilution factor to reduce the matrix effect and achieve an appropriate pre-concentration factor. As a result, we chose redisolution volumes of 1 mL for river samples and 3 mL for sewage waters. These volumes provided about 20% of the matrix effect in river water and sewage.

3.3 Method validation

In order to validate the method, we evaluated the linear range, the LOD, the LOQ, the repeatability (expressed as relative standard deviation) and the recoveries for glucocorticoids under optimized SPE conditions in the three kinds of water samples studied. River water, influent and effluent sewage were spiked at a level close to the limit of quantification. The spiked level was 10, 20 and 30 ng/L for river water, influent and effluent sewage respectively and analysis was carried out in triplicate.

The method was linear in the studied range (LOQ-100 ng/L for river, LOQ-600 ng/L for effluent and LOQ-1,500 ng/L for influent). Limits of Detection (LOD) and Quantification (LOQ) were obtained experimentally. The analyte concentrations for the method LOD corresponded to an analyte signal of approximately 3 times when these was compared with the noise signal obtained in a region near to the analyte signal and the method LOQ

corresponded at the lower point into the liner range of calibration curve. These limits were obtained from all three matrices and were equal for all the glucocorticoids (0.5 ng/L and 1.5 ng/L in river water, 3 ng/L and 10 ng/L in effluent sewage and 7.5 ng/L and 20 ng/L in influent sewage), except triamcinolone acetonide, whose limits were higher (1.5 ng/L and 5 ng/L in river water, 9 ng/L and 20 ng/L in effluent sewage and 20 ng/L and 50 ng/L in influent sewage). This is due to their different signal response, as discussed in section 3.1. These limits are similar to those reported in previous methods [17,19,24,25]. Only methods described by Hong Chang *et al.* [5,23] achieved lower LOD and LOQ since their preconcentration factor is higher, but this method is more laborious because it includes several clean-up steps.

Analyte recoveries were calculated with a matrix calibration curve obtained from a set of blank samples fortified after the SPE procedure at different concentration levels. This operational mode ensured that the concentration required in the calibration curve was real and eliminated the signal differences between the standards and samples caused by the matrix effect. Also a blank sample was analysed in order to subtract the possible signal of existing analytes (cortisone and hydro-cortisone appeared at a significant level in the sample blanks). All recoveries exceeded 82% and in most cases were between 90 and 99%. Relative Standard Deviation (%RSD) was always less than 8% and was commonly found between 1 and 5%. All validation parameters for the nine glucocorticoids are shown in Table 4.

4 Application to environmental samples

The SPE/UHPLC-MS/MS method developed was used to determine the nine glucocorticoids in various river waters and in influent and effluent sewages. These results show that the river waters did not contain glucocorticoid residues. However, glucocorticoids were frequently detected in the sewage samples, often between the LOD and LOQ. The results are shown in Tables 5 and 6.

In influent sewage four glucocorticoids were determined at 20 to 300 ng/L (prednisone, prednisolone, cortisol and cortisone) and four more were found above the LOD (methylprednisolone, betamethasone, dexamethasone and triamcinolone acetonide). In different samples of effluent sewage we found five glucocorticoids between the LOD and LOQ. Flumethasone was not detected in any sewage sample. In all cases, the presence of these compounds in the different samples was corroborated by two qualifier ion ratios and their retention time, as can be seen in Tables 5 and 6 under requirements of Commission Decision 2002/657/EC [34]. An example of the MRM chromatograms obtained for influent sewage is shown in Figure 3.

The study of glucocorticoids residues in influent and effluent sewage

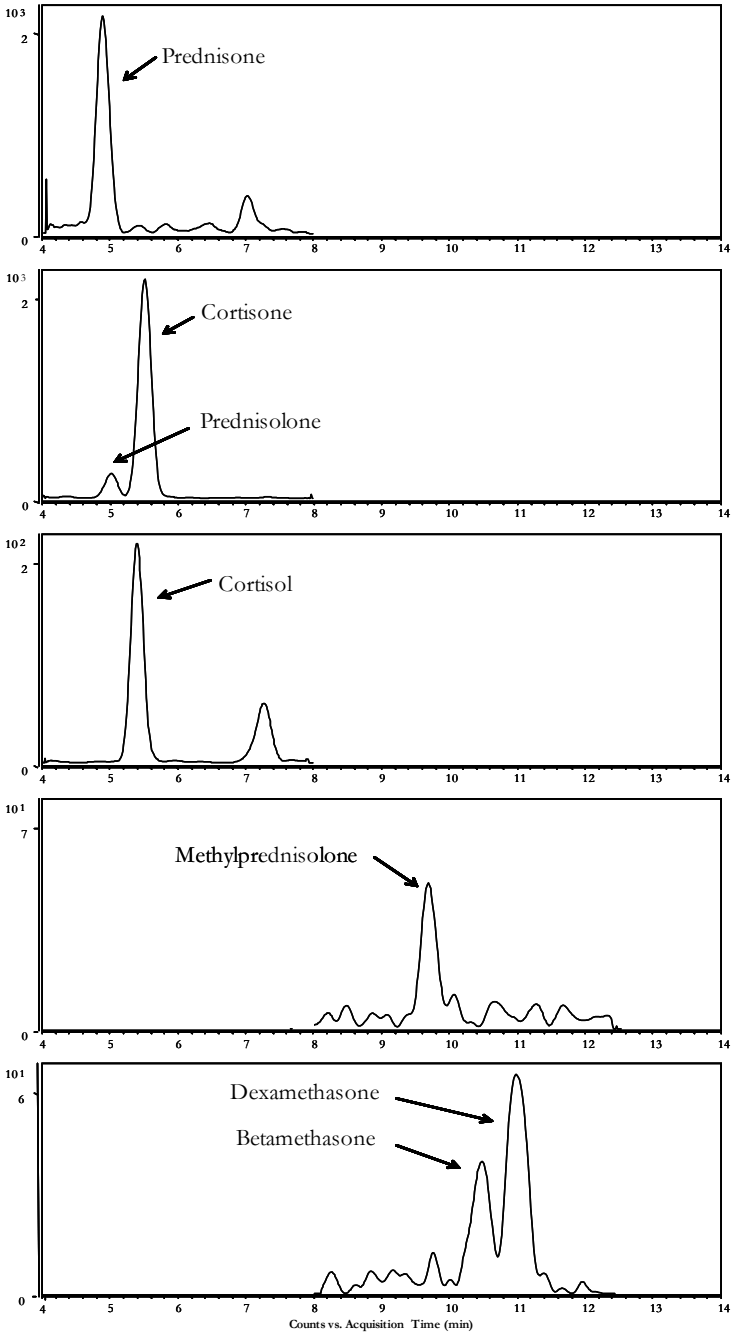


Fig. 3. MRM chromatogram obtained of influent sewage collected in STP 2 in February 2011.

showed that the treatments received in the two STPs under study (activated sludge biological treatment) are quite effective. The concentration found in influent sewage was reduced in effluent sewage to low ng/L levels (<LOQ). However, these low levels do not allow an objective assessment of their elimination ratio and, unfortunately, on a couple of occasions glucocorticoid residues were still detected in effluent sewages at levels close to the LOD.

As expected, the highest levels of glucocorticoids corresponded to endogenous glucocorticoids (cortisol and cortisone). Nevertheless, two synthetic glucocorticoids (prednisone and prednisolone) were detected in influent sewage at significant concentrations when compared with the cortisol or cortisone results.

The results coincide with other studies in the EU and Asia, which indicates that the presence of glucocorticoid residues in river waters is negligible since only cortisone (up to 2.67 ng/L) and hydrocortisone (up to 1.9 ng/L) have been detected in rivers in China [17] and Hungary [25], respectively. Moreover, a study in urban rivers in China [23] demonstrated that river samples near to STPs discharging sites contain several glucocorticoids residues. A large number of glucocorticoid residues were detected at very low levels when sewage samples were analyzed in different countries, as is reported in the literature [5,17,19,22,24]. The most commonly detected glucocorticoids are cortisone and hydrocortisone in influent sewage. Nevertheless, they are reduced when they pass through to STPs.

5 Conclusions

The chromatographic separation of two epimers (betamethasone and dexamethasone) should be highlighted since both have been detected in certain influent and effluent sewages which had not been studied previously. This should help provide a better understanding of the presence of these compounds in environmental water samples.

We have successfully developed an analytical method based on SPE preconcentration and UHPLC-MS/MS to determine nine glucocorticoids in river waters and influent and effluent sewages at low ng/L levels in less than 13 min.

The recoveries for glucocorticoids in water samples were close to or above 90% at levels near to the LOQ in all the water types. The repeatability was less than 8% in terms of relative standard deviation.

The method was then used to determine these drugs in three rivers and two STPs in Catalonia. The most commonly found glucocorticoids were prednisone, prednisolone, hydrocortisone and cortisone in influent sewages, although other glucocorticoids were detected below the LOQ in some samples.

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UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
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3.1.2. Pressurised liquid extraction and ultra-high performance liquid chromatography-tandem mass spectrometry to determine endogenous and synthetic glucocorticoids in sewage sludge

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PRESSURISED LIQUID EXTRACTION AND ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY TO DETERMINE ENDOGENOUS AND SYNTHETIC GLUCOCORTICOIDS IN SEWAGE SLUDGE

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Abstract

Glucocorticoids are a type of steroid hormones classified as endocrine disrupting chemicals (EDCs) and they are widely used by human and animal medicine. In this paper, we describe the development and validation of an analytical method for the determination of nine selected glucocorticoids (betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide) in sewage sludge by pressurised liquid extraction (PLE) and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). PLE with on-cell clean-up followed by solid-phase extraction (SPE) clean-up (Oasis HLB) has been applied to extract the analytes and clean up the matrix. Recoveries of the method were from 8% for prednisone and cortisone to 73% for triamcinolone acetonide. The developed method achieves limits of detection (LODs) between 1-5 μg/Kg dry weight (d.w.) in sludge and the repeatability expressed as relative standard deviation (%RSD, 50 μg/Kg (d.w.), n=3) is less than 8%. The PLE/SPE/UHPLC-MS/MS method was successfully applied to determine these pollutants in sludge samples from two sewage treatment plants located in the Tarragona area. Cortisone was found at levels below LOQ, cortisol between 5.2 and 6.1 μg/Kg (d.w.) and prednisolone between <LOQ and 6.0 μg/Kg (d.w.).

***Keywords**: Glucocorticoids; Pressurised liquid extraction; Sludge; Ultra-high performance liquid chromatography; Tandem mass spectrometry.*

1 Introduction

A lot of pharmaceuticals are discharged into the environment without any concern from the world's population. However, in recent years it has become a crucial issue since the danger to human health from chronic exposure to some of these compounds has been proven [1]. Pharmaceuticals used in human and animal medicine are a very heterogeneous class of emerging organic pollutants and they have been determined in several environmental matrices such as sewage [2,3], ground water [4] or marine sediments [5].

The main intrusion route of these compounds into aquatic systems is the excretion of free or conjugate form of pharmaceuticals by either humans or animals. The pharmaceuticals arrive via this route to sewage treatment plants (STPs) and several studies show that conventional treatments used are not fully effective at eliminating them [6]. The fate of these pharmaceuticals during sewage treatment is a partially adsorption onto activated sludge. This can result in the transfer of these pollutants to rivers and oceans directly via effluent sewages or in the contamination of agricultural soils as a result of using contaminated sludge from STPs as manure.

Synthetic glucocorticoids are a large group of drugs intended for human or veterinary use with a wide range of therapeutic applications, such as combating asthma, skin allergies or inducing labour. Glucocorticoids are a sub-group of steroids with significant physiological functions and, therefore, their ecotoxicological risk is apparent. Studies in fish have shown that long-term treatment with cortisol causes adverse effects to locomotion, inhibits aggressive behaviour, influences the immune response and may also affect the sexual behaviour of fish [7,8]. Unfortunately, they have a significant effect as growth promoters and, therefore, are banned in the EU for this use. Maximum residue levels (MRLs) were established in 1990 in order to eliminate bad farming practices (EU nº 37/2010) [9]. As a result, numerous studies have been published lately related to their determination in biological matrices such as plasma, liver, eggs and milk [10-16]. However, only a few articles refer to their determination in environmental matrices. To our knowledge, there are published papers that report methods to determine some glucocorticoids in surface waters [17-20], sewage [17,19,21-25], sewage sludge [17,25], sediments [26] and soils [26,27]. To date, however, evidence of the presence of glucocorticoids has only been reported in river water [17,18] and sewage [17,19,21-23]. Therefore, sewage sludge is a possible destination for these steroids, due to their removal in sewage treatment by activated sludge. However, in-depth information about their occurrence and fate in sludge is not available.

Various analytical extraction techniques have been used to extract different types of steroids from solid samples such as soil or sludge. The

conventional Soxhlet extraction and ultrasonic solvent extraction (USE) [17] are the most commonly used techniques because they do not involve expensive equipment, but they often require large volumes of solvent and time. As a result, in recent years, extraction techniques such as microwave-assisted extraction (MAE) [28] or pressurised liquid extraction (PLE) [27] have been widely accepted, due to reduced time and solvent consumption as well as the possibility of the automation of extraction and hence improved reproducibility.

To determine glucocorticoids, liquid chromatography combined with tandem mass spectrometry [21], fluorescence [29] or UV-Vis [30] are the most frequently used detection systems. In addition, gas chromatography [31] or capillary electrophoresis [32] have also been used as separation techniques. In recent years, ultra-high performance liquid chromatography (UHPLC) has been established as a day-to-day technique because of its capacity to provide faster analysis and better efficiency in the separation of compounds. These benefits combined with the specificity, sensitivity and selectivity that tandem mass spectrometry (MS/MS) provides make UHPLC-MS/MS the most suitable technique for determining these emerging organic pollutants in environmental matrices.

In the present paper, we develop an analytical method for the simultaneous determination of nine corticosteroids, endogenous (cortisol and cortisone) and synthetics, in sewage sludge by using PLE followed by UHPLC-MS/MS analysis. Several parameters that affect the extraction procedure have been evaluated and, moreover, different clean-up strategies (on-cell clean-up and solid-phase extraction) have been evaluated in order to prevent ion suppression in mass spectrometry when the sludge extracts are analysed.

2 Experimental

2.1 Reagents and standards

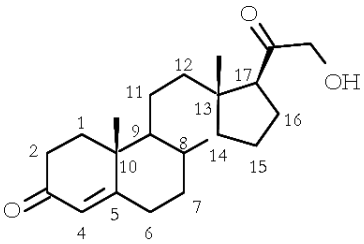
All the standards: betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, dichlorisone acetate, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide were purchased from Sigma-Aldrich (St. Louis, USA). The chemical structure of glucocorticoids is presented in Figure 1. Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and stored at -20 ºC. Fresh stock solutions of 100 mg/L of each compound in methanol were prepared every month and stored at 4 ºC. A mixture of all compounds in water/acetonitrile (4:1) at a concentration of 1 mg/L was prepared weekly and working solutions were prepared daily by diluting these solutions with water/acetonitrile (4:1) or acetone.

Ultrapure water was obtained with an ultrapure water purification system from Veolia waters (Sant Cugat del

Vallés, Spain). Acetone, acetonitrile (ACN), methanol (MeOH), n-hexane and ethyl acetate (EtOAc) were of HPLC grade from SDS (Peypin, France) and nitrogen gas was from Carburos Metálicos (Tarragona, Spain). Formic acid from Merck (Darmstadt, Germany) was used to adjust the pH of the mobile phase and diatomaceous earth used in PLE was purchased from Sigma-Aldrich.

2.2 Instrumentation and equipment

The chromatographic system was an Agilent 1200 series (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). It was equipped with an automatic injector, a degasser, a binary pump, and a column oven. The chromatographic column was a Zorbax Eclipse XDB-C_{18} (50 x 4.6 mm, 1.8 μm) (Agilent Technologies, Waldbronn, Germany). The freeze-drying system was supplied by Labconco (MO, USA) and PLE was carried out with an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA). A 200 mg Bond Elut Plexa (Varian, Agilent Technologies) connected to a manifold (Teknokroma, San Cugat del Vallés, Spain) with a pump as a vacuum source was used for the SPE procedure.



Compound		1-2	6	9	11	16	17	16-17
Betamethasone	BMS	=		F	OH	CH_3	OH	
Cortisol	HCOR				OH		OH	
Cortisone	COR				=O		OH	
Dexamethasone	DMS	=		F	OH	CH_3	OH	
Flumethasone	FMS	=	F	F	OH	CH_3	OH	
Methylprednisolone	MPNL	=	CH_3		OH		OH	
Prednisolone	PNL	=			OH		OH	
Prednisone	PNS	=			=O		OH	
Triamcinolone acetonide	TACA	=		F	OH			$OC(CH_3)_2O$

Fig. 1. Chemical structure of glucocorticoids.

2.3 Sampling and sample preparation

The sewage sludge samples were collected from two domestic sewage treatment plants (STPs) provided with an activated sludge biological treatment. Both plants are located in the area of Tarragona and each one treats sewage from a population greater than 100,000 inhabitants. STPs receive urban wastewaters and some industrial discharges. The average flow rates are 30,000 m^3/day (STP1) and 16,000 m^3/day (STP2) and the biological oxygen demand (BOD_5) is 400 mg/L for both treatment plants. The sewage sludge samples corresponded to a mix of primary and secondary sewage, which was anaerobically digested and then dehydrated using press filters. Sludge was frozen before being lyophilised and then was crushed in a mortar and pestle and sieved (125 μm) to obtain particles with the same diameter.
Spiked samples were prepared by adding the stock mixture of standards in acetone (the required volume to wet and cover the sludge). The solvent was slowly evaporated at room temperature inside an extractor hood with frequent homogenisation of the sample throughout the two days prior to extract.

2.4 Pressurised Liquid Extraction

One gram of freeze-dried sample was placed into 11 mL stainless steel extraction cell and mixed with 1 g of diatomaceous earth. The PLE method included two steps: the first step was a defatting of the sample with n-hexane. The extraction was performed at 40 ºC and 1,500 psi with a preheating period of 5 min, two cycles of 1 min, flush volume of 100% of cell volume and nitrogen purge of 360 s. This extract was rejected. The second step was the extraction of analytes with a methanol:acetone (80:20) mixture at 40 ºC and 1,500 psi. Operational conditions were as follows: preheating period of 5 min, one cycle of 10 min, flush volume of 30% of cell volume and nitrogen purge of 120 s. The methanol:acetone extract was evaporated to dryness under a flow of N_2 and the residue was redissolved with 25 mL of water prior to further clean-up with a SPE procedure.

2.5 SPE clean-up

To decrease the ion suppression resulting from a high matrix effect, a SPE procedure was applied after the PLE of the sludge. SPE was carried out using 200 mg Bond Elut Plexa cartridges. These were preconditioned with 5 mL of MeOH followed by 5 mL of ultrapure water. The PLE extract, redissolved in 25 mL of water, was loaded into the cartridge which was slightly dried under vacuum and washed with 5 mL of ultrapure water. Before the elution step, the sorbent was further dried under vacuum and then the analytes were eluted from the cartridge with 10 mL of MeOH. The eluate was concentrated under a flow of nitrogen gas to dryness and the residue was

redissolved in 1 mL of water:acetonitrile (4:1) and filtered with a 0.22 μm nylon filter before UHPLC-MS/MS analysis.

2.6 UHPLC-MS/MS analysis

The UHPLC-MS/MS method was developed in a previous paper [19]. Briefly, chromatographic separation of analytes was performed with a gradient elution. Solvent A was water:acetonitrile (78:22, v:v) with formic acid (0.1 %) and solvent B was methanol:acetonitrile (78:22, v:v) with formic acid (0.1%). The gradient started at 0.8% of solvent B, then increased to 5% at 5 min, 15% of B at 11.5 min, 50% at 12 min and then kept constant for 1.5 min more. Finally, the gradient was increased to 99.9% at 14 min and remained constant for 0.5 min before returning to initial conditions over 0.5 min. All the compounds eluted within 11.3 min. The chromatographic system operated at 50 ºC to reduce the back pressure and decrease the retention factor of analytes and, in addition, the flow rate was 1 mL/min to reduce the time per run. Injection volume was 50 μL.

ESI interface and MS/MS conditions were individually optimised for each compound with a flow injection of a standard solution of each compound. The average conditions selected for the optimum performance of the ESI in the negative mode were: nebuliser pressure 40 psi, drying gas (N_2) flow rate 12 L/min, drying gas temperature 350 ºC, and capillary voltage 2,000 V. Cone voltage and collision energies for each compound are described in Table 1. Chemical structure of each product ion is proposed in a previous paper [19].

Table 1. UHPLC-MS/MS acquisition parameters in MRM mode.

Compound	t_R (min)	CV (V)	Parent ion (m/z)	Daughter ions (m/z)		
Prednisone	3.7	100	403	**327 (10)**	299 (15)	285 (30)
Prednisolone	3.8	100	405	**329 (10)**	280 (30)	295 (30)
Cortisol	4.1	100	407	**331 (10)**	297 (35)	282 (35)
Cortisone	4.2	100	405	**329 (10)**	301 (15)	311 (30)
Methylprednisolone	7.3	110	419	**343 (10)**	294 (35)	309 (35)
Betamethasone	7.7	110	437	**361 (15)**	307 (35)	292 (35)
Flumethasone	7.9	110	455	**379 (15)**	305 (35)	325 (30)
Dexamethasone	8.2	110	437	**361 (10)**	307 (35)	292 (35)
Triamcinolone acetonide	11.3	110	479	**413 (15)**	337 (20)	375 (10)

CV is cone voltage.
Collision Energies are in brackets (eV).
The most intense daughter ion for each compound is in bold.

3 Results and discussion

3.1 UHPLC-MS/MS

The UHPLC-MS/MS method was developed in a previous paper in which the studied glucocorticoids were determined in water samples from rivers and sewage [19]. The chromatographic method uses a gradient elution with a ternary mobile phase (water, acetonitrile and methanol). The use of methanol into the ternary mobile phase allows a slight chromatographic separation of prednisone/prednisolone and cortisone/cortisol couples (set of polar compounds) and the separation between betamethasone and flumethasone compounds. In addition, the acetonitrile allows the chromatographic separation of the epimers betamethasone and dexamethasone.

Moreover, the use of methanol provides good analyte desolvation in the electrospray interface and we can therefore work at an elevated flow rate (1 mL/min). This flow rate reduces the run time (the compound with the highest retention elutes in 11.3 min) without losses in column efficiency because they are composed of sub-2 μm particles. The run time is not as short as would normally be expected for an UHPLC analysis due to the necessary separation of epimers which cannot be differentiated by MS/MS.

A comprehensive study of the optimisation of several parameters of the tandem mass spectrometer was also carried out in the previous paper [19]. It was observed that the negative ionisation mode provided more intense MRM transitions than positive ionisation mode. Only triamcinolone acetonide had less intense MRM transitions in negative mode because its fragmentation pathways were slightly different from other glucocorticoids under study. Assigned fragments for all analytes and their cone voltages and collision energies are described in Table 1.

3.2 Pressurised liquid extraction

To achieve a fast and efficient extraction of the target compounds from a solid matrix using a PLE system, several operational parameters must be optimised. The solvent, temperature, time and number of cycles should therefore be tested. Pressure, flush volume and purge time can also be optimised, but it is well known that these parameters do not have a significant effect on the extraction efficiency.

Literature reports a methanol/ acetone mixture [27] or pure ethyl acetate [17] as the most suitable solvents for the extraction of glucocorticoids from environmental solid samples. Thus, water, methanol, acetone, ethyl acetate and n-hexane were tested in the first instance, as pure solvents or as mixtures between them. Other initial conditions were as follows: 60 ºC, 5 min to preheat cell, 5 min of static time, 1,500 psi of pressure, 1 cycle, 30% flush volume and 120 s of purge time. Lyophilised sludge samples were spiked at high concentration (1 mg/kg (d.w.)) for

each compound to ensure that there is no possibility of the matrix interferences affecting the accurate quantification of analytes. For optimisation purposes, extracts with water-miscible solvents (15-20 ml approx.) were brought up to 50 mL of final volume with ultrapure water to prevent ion suppression and achieve similar conditions of the mobile phase prior to injecting them into the UHPLC system. The extracts containing ethyl acetate and/or hexane were evaporated to dryness under nitrogen flow and the residues were redissolved with about 15 mL of methanol and then brought up to 50 mL with ultrapure water.

An important aspect to be considered is how the sludge was spiked (procedure is further described in section 2.3). We observed experimentally that the recoveries of these compounds are highly dependent of the contact time between the matrix and analytes before extraction process because when this time was shorter (some hours) the recoveries were close to 100%. Consequently, we carried out a batch of experiments with the extraction conditions stated above and acetone as extraction solvent to determine the time necessary to obtain constant recoveries along the time. The experiments led us to wait two days prior to extraction.

Results showed (see Tables 2 and 3) that the methanol:acetone mixtures were the most efficient for extracting the target analytes from sludge, rather than pure methanol or acetone. Water or ethyl acetate were also capable of extracting the glucocorticoids from sludge, but always with a lower efficiency than methanol/acetone mixtures. N-hexane could not extract any compound due to its low polarity. In methanol:acetone mixtures, recoveries were between 13% and 30% for more polar compounds (prednisone, prednisolone, cortisol and cortisone), between 25% and 49% for medium-polar compounds (methylprednisolone, betamethasone, dexamethasone and flumethasone) and between 62% and 74% for the non-polar compound (triamcinolone acetonide). It is notorious that the two compounds with a ketone group at position C11 (prednisone and cortisone), rather than an alcohol group like the other glucocorticoids, showed much lower recoveries than the other compounds. The results show that a mixture rich in methanol (80% in volume) is able to enhance the efficiency of extraction of triamcinolone acetonide from 60% in rich acetone mixtures (80%) to 74% in rich methanol mixtures (80%). For the rest of the compounds, no significant differences in their extraction were found. Methanol:acetone (80:20) was therefore chosen for the PLE process.

Regarding the extraction temperature, a range from room temperature (~25 ºC) up to 100 ºC was studied with a mixture of methanol:acetone (80:20). The other extraction conditions were the same as described previously. A temperature of 40 ºC provided the best recoveries for all analytes (between 16% and 39% for more

polar analytes, between 34% and 56% for medium-polar and 90% for the non-polar). These recoveries were better than at room temperature. The recoveries decreased when temperature was increased to 60 ºC or 80 ºC and were below 10% for the majority of compounds when the temperature was 100 ºC. This fact is probably due to a thermal decomposition of analytes and may indicate that, at 40 ºC, the thermal decomposition of compounds is very low.

Static extraction time and number of cycles were also studied in order to enhance the efficiency of extraction. Static times of 3, 5, 10 and 20 minutes were studied. A static time of 10 minutes resulted in a slight improvement of the extraction process (recoveries were between 19% and 43% for more polar, 41% and 65% for medium-polar and 90% for non-polar). A time of 20 minutes did not result in a significant increase in the extraction efficiency. Regarding to the number of cycles, 1, 2 and 3 cycles with a static extraction time of 10 minutes were tested. Two cycles of extraction only improved recoveries by 2% for some compounds and then, increase the number of cycles was discarded. Therefore, one cycle and a static time of 10 minutes were chosen as optimal parameters for the PLE extraction.

In addition, the fact that n-hexane does not extract the compounds from sludge was exploited in order to carry out an on-cell matrix clean-up. Thus, we extracted the insoluble-water interfering substances such as fat and oil before the extraction of analytes with the methanol:acetone mixture. A much cleaner extract was obtained and differences on the recovery of analytes were not observed when this on-cell clean-up was applied.

Finally, it was decided to concentrate the PLE extract in order to minimise the dilution of the sample and therefore improve the method detection limits. Unfortunately, the final extract was extremely dark and turbid when evaporated residues were redissolved with small volumes of water:acetonitrile (4:1) and filtered (0.22 μm). Therefore, to improve the cleaning of the sample, reduce the matrix effect and minimise the final volume of dilution, a SPE clean-up was applied prior to analysis by UHPLC-MS/MS.

3.3 SPE clean-up

Our prior knowledge in the determination of glucocorticoids in sewage and river waters led us to choose a polymeric sorbent for SPE which is capable of retaining both polar and non-polar compounds. The Bond Elut Plexa (Hydroxylated surface and polystyrene-divinyl-benzene core with a macroporous structure) was selected as the most suitable for this purpose based on the excellent results obtained in a previous study [19]. This sorbent was applied for the SPE of glucocorticoids in sewage and it provided recoveries above 90% for all compounds in this matrix. Subsequently, a similar strategy was applied to the sludge extracts. The

PLE extracts were then evaporated under a stream of nitrogen and were subsequently redissolved with about 25 mL of ultrapure water to load into the cartridge. Other considerations of the SPE procedure are extensively described in Section 2.5.

In order to minimise the sample interferences without involving significant losses in the recovery of the analytes, different washing stages were tested in the SPE clean-up step, including 5 mL of ultrapure water, 5 mL of water:methanol (90:10, v:v) and 2 mL of n-hexane. Sludge extracts were spiked at 50 μg/L for each compound after the PLE extraction and evaporation process and were used for the SPE experiments. Recoveries were calculated by comparing the signal obtained by the SPE extracts with a sludge extract subjected to the same SPE process, spiked after the elution step. Moreover, the signal obtained was compared with a standard in order to evaluate the matrix effect. The recoveries associated at the SPE with the different washes are shown in Table 4.

Table 2. PLE recoveries (%) obtained with different pure solvents.

Compound	Water	Methanol	Acetone	Ethyl acetate
Prednisone	8	10	6	2
Prednisolone	14	13	12	7
Cortisol	7	5	4	2
Cortisone	13	24	12	11
Methylprednisolone	17	21	22	11
Betamethasone	24	12	24	7
Flumethasone	10	8	14	6
Dexamethasone	14	12	17	11
Triamcinolone acetonide	33	20	31	24

Sludge was spiked at 1 mg/Kg (d.w.) and %RSD(n=3)<10%.
Extraction conditions are described in section 3.2.

The recoveries obtained when ultrapure water was used in the washing step were between 80% and 100% depending on the compound. These results are similar to those obtained for sewage and river waters [19] and indicate that the matrix did not decrease analyte-sorbent interaction. This indicated that the properties of the sorbent were adequate for these analytes in this matrix. Regarding the washing step experiments, washing with water:methanol gave the worst results of the three washes (recoveries between 70 and 80 %) due to the analytes being partially eluted in this step. Hexane wash did not affect the recovery of polar and medium-polar compounds (when these are compared with water wash results), but the recovery of most non-polar compounds, such as triamcinolone acetonide, decreased from 95% when water was used to 77% with hexane. In addition, results showed that all of

the different washes involved a matrix effect below 20% for all compounds. A wash step with ultrapure water was therefore selected based on these results (recoveries and clean-up) as well as for the sake of simplicity.

3.4 Method validation

The method validation involved the evaluation of the linear range, LOD, LOQ, repeatability and reproducibility between days (expressed as % Relative Standard Deviation) and recoveries for glucocorticoids under the optimised sample extraction procedure (PLE and SPE). All these parameters were calculated with a sludge sample which did not contain the glucocorticoids and are shown in Table 5.

To calculate the recoveries, a calibration curve obtained from a set of posteriori (after PLE and SPE) fortifications (between 1 and 100 μg/Kg (d.w.), except for triamcinolone acetonide, between 5 and 100 μg/Kg (d.w.)) of blank sludge samples was used. Then, three blank sludge samples were spiked at 50 μg/Kg (d.w.), analysed by the entire method and recoveries were calculated by the calibration curves already mentioned. Recoveries of the analytes had a higher variation range depending on their polarity, as shown in the results. Thus, the group of high-polar compounds showed poor recoveries in a range from 8% to 20%. For the medium-polar compounds that range increased from 28% to 43% and the low-polar

Table 3. PLE recoveries (%) obtained with different solvent mixtures.

Compound	MeOH/Acetone (20:80)	MeOH/Acetone (50:50)	MeOH/Acetone (80:20)	MeOH/EtOAc (50:50)	Acetone/EtOAc (50:50)	MeOH/Acetone/EtOAc (35:35:30)
Prednisone	13	13	15	12	5	9
Prednisolone	24	27	28	20	8	17
Cortisol	25	27	30	7	3	6
Cortisone	13	13	13	27	12	20
Methylprednisolone	25	26	28	37	14	23
Betamethasone	47	49	48	29	12	21
Flumethasone	34	34	31	18	7	12
Dexamethasone	32	33	34	22	9	16
Triamcinolone acetonide	62	68	74	42	23	36

Sludge was spiked at 1 mg/Kg (d.w.) and %RSD(n=3)<10%.
Extraction conditions are described in section 3.2.

compound their recovery increased up to 73%.

Although the matrix effect had been reduced significantly, it was decided to use a matrix matched calibration curve (with sludge samples spiked before PLE) for the quantification of analytes in order to obtain more accurate results and also correct the recoveries. However, it would be possible to use an external calibration curve for a quick and easy quantification of the glucocorticoids with the developed method because the ion suppression was less than 20%. We also discarded the use of an internal standard to correct ion suppression due to its low commercial availability and high price.

Table 4. SPE recoveries (%) obtained with different washing steps.

Compound	Water	Water/MeOH (9:1 v/v)	n-hexane
Prednisone	76	73	85
Prednisolone	77	72	87
Cortisol	108	83	101
Cortisone	100	82	100
Methylprednisolone	81	82	89
Betamethasone	91	86	88
Flumethasone	81	74	74
Dexamethasone	83	74	88
Triamcinolone acetonide	96	82	77

Sludge extracts (~25 mL) were spiked at 50 μg/L.
SPE procedure was described in section 2.6.
%RSD (50 μg/L, n=3) < 8%.

Dichlorisone acetate, a compound of the same family, was proposed in some papers for this purpose [12]. However, this option does not have a total guarantee that a compound of the same family to the target analytes was not present in samples. A major discussion of this issue was included in a previous paper [19]. Linear range, limit of quantification (LOQ) and detection (LOD) were obtained experimentally by spiking sludge at different levels prior to the extraction procedure. These parameters were equal for all glucocorticoids except triamcinolone acetonide, for which the parameters were higher than the others. This is due to their lower signal response, as previously discussed. The linear range started at LOQ (defined as the lowest calibration point) and went up to 100 μg/Kg (d.w.) with good linearity for all compounds (R^2>0.997). The analyte concentrations for LODs were corresponded to a signal/noise ratio equal to 3 times. Thus, LOQ were 1 μg/Kg (d.w.) and LOD were 0.5 μg/Kg (d.w.) for all glucocorticoids except triamcinolone acetonide (for which LOQ was 5 μg/Kg (d.w.) and LOD was 1 μg/Kg (d.w.)).

Table 5. Validation values for PLE/SPE/UHPLC-MS/MS method.

Compound	Recovery[a] (%)	Repeatability[b] (%RSD, n=3)	Reproducibility[c] (%RSD, n=3)	LOD (µg/Kg (d.w.))	LOQ (µg/Kg (d.w.))
Prednisone	8	1	2	0.5	1.0
Prednisolone	18	1	3	0.5	1.0
Cortisol	20	4	6	0.5	1.0
Cortisone	8	2	3	0.5	1.0
Methylprednisolone	43	6	9	0.5	1.0
Betamethasone	43	6	9	0.5	1.0
Flumethasone	28	1	4	0.5	1.0
Dexamethasone	28	3	6	0.5	1.0
Triamcinolone acetonide	73	8	10	1.0	5.0

[a] Recoveries calculated by a 50 µg/Kg (d.w.) spiked sludge.
[b] Intra-day repeatability calculated as %RSD by a 50 µg/Kg (d.w.) spiked sludge.
[c] Day-to-day reproducibility calculated as %RSD by a 50 µg/Kg (d.w.) spiked sludge.

Repeatability (intra-day) and reproducibility (day-to-day) were obtained with three replicates of a sample spiked at 50 µg/Kg (d.w.). Repeatability (%RSD, n=3) was always less than 8% and was commonly found to be between 1% and 4%. Reproducibility between days was always less than 10% (%RSD, n=3). A MRM chromatogram obtained from a spiked sludge at 10 µg/Kg (d.w.) is shown in Figure 2.

In general, recoveries were not very high, but linearity was good and the method limits of quantification and detection were reasonably good in comparison with previous studies for this type of matrix and analytes. Previously, Liu *et al.* [17] developed a method to determine five glucocorticoids in sludge with an ultrasonic solvent extraction with ethyl acetate. The LODs (0.8-2.06 µg/Kg (d.w.)) and LOQs (1.95-6.86 µg/Kg (d.w.)) achieved with this method were slightly higher than in our study. Moreover, Fan *et al.* [25] describe a method to determine seven glucocorticoids in sludge based on ultrasonic solvent extraction with methanol:acetone (50:50) followed by three SPE clean-up stages (HLB followed by a NH_2-SPE and finally, with a florisil (or silica) cartridge). The LODs achieved with this method (0.02-0.2 µg/Kg (d.w.)) are slightly better than those from our study but the LOQs were not reported.

In addition, two other papers determined the glucocorticoids in environmental solid samples such as soils [26] and sediments [26,27]. In these two papers, a PLE method for the extraction of glucocorticoids from samples was applied. Gineys *et al.* [27] reported recovery values similar to those found in our study but only two glucocorticoids were determined and the method was only applied to

spiked soil samples (PLE extraction with methanol:acetone (50:50) at 80 ºC). Pérez-Carrera *et al.* [26] in a study into the determination of 32 human and veterinary residues detected the presence of prednisolone (below LOQ) in one sediment sample.

4 Application to environmental samples

The PLE/SPE/UHPLC-MS/MS method was applied to determine glucocorticoids in eight sludge samples from the two STPs over a period of seven months and only three samples contained low residues of glucocorticoids (Table 6). The presence of these compounds in the different samples was corroborated by their retention time and two qualifier ion ratios, as can be seen in Table 6 under requirements of Commission Decision 2002/657/EC [33].

Thus, hydrocortisone was determined to be more frequent, between 5.2 and 6.1 μg/Kg (d.w.) in three samples taken from the two urban sewage treatment plants. Prednisolone and cortisone were also determined in few samples but their concentrations were lower than hydrocortisone. Prednisolone only appeared in one sample at 6 μg/Kg (d.w.) and in another sample, prednisolone and cortisone were found below LOQ. The other glucocorticoids were not detected in any of the sludge samples analysed. The presence of hydrocortisone in the sludge was expected since hydrocortisone has a natural origin and its concentration levels may therefore be expected to be higher than the synthetic glucocorticoids.

Table 6. Concentrations (μg/Kg (d.w.)) and qualifier ion ratios (q/Q) in sewage sludge samples.

Compound	S1 (STP_1)	S2 (STP_2)	S3 (STP_2)	q_1/Q (%)	q_2/Q (%)
Prednisolone	n.d.	<LOQ	6.0 (22,11)	20	9
Cortisol	5.2 (17,15)	6.1 (18,16)	5.6 (19,16)	16	15
Cortisone	n.d.	<LOQ	n.d.	21	8

Qualifier ion ratios (%) in samples are in brackets

However, the presence of prednisolone should be highlighted due to its synthetic origin (exogenous). Prednisolone is usually detected in influent sewage at a level lower than hydrocortisone or cortisone. However, the concentration of prednisolone (together with prednisone) in influents is the most elevated of synthetic glucocorticoids [19].

These results demonstrate that activated sludge is one destination of glucocorticoids. Despite this, their elimination from the sewage is not fully complete as shown in previous studies [19]. In addition, glucocorticoids remain into the sludge and if used as manure, it may lead to new forms of contamination of the environment.

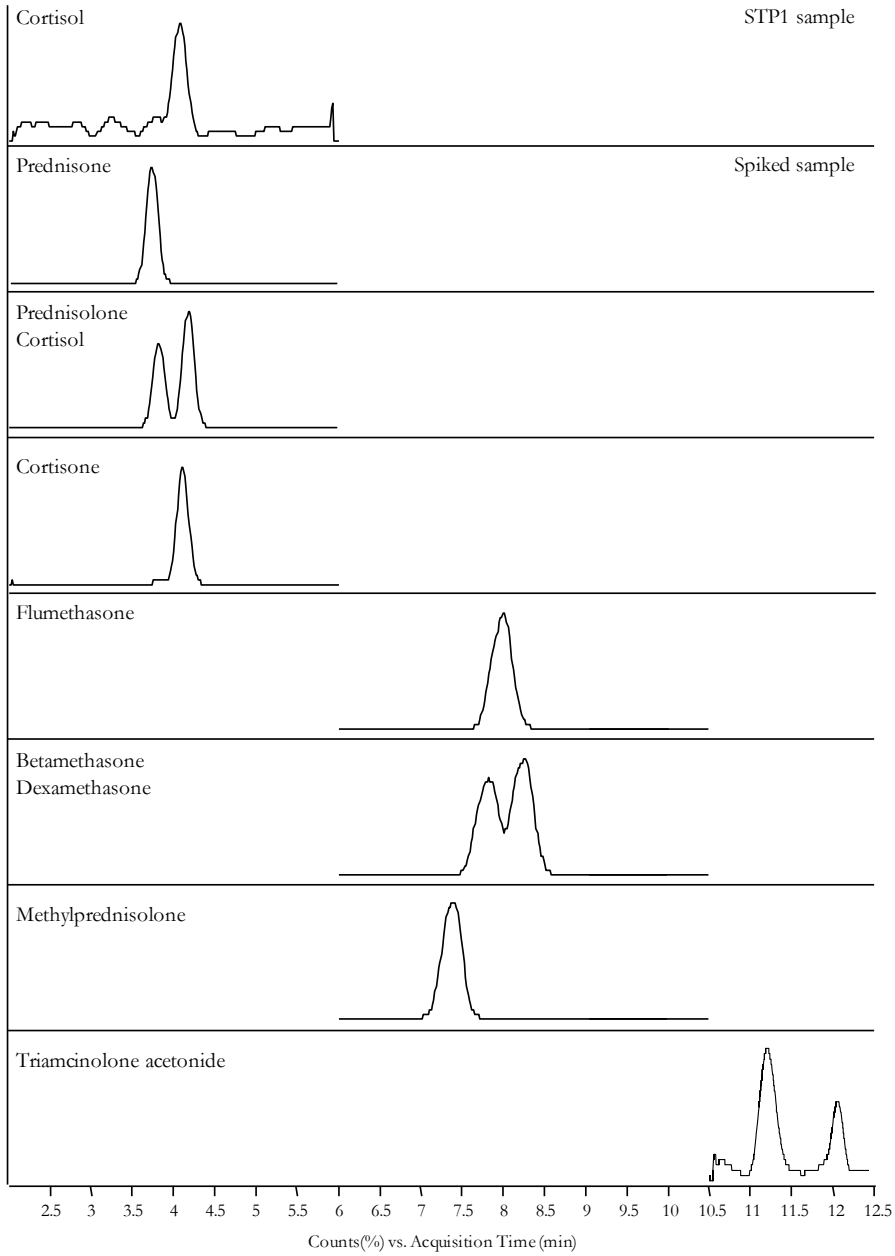


Fig. 2. Quantification MRM trace of cortisol detected in one sample from STP1 and MRM chromatogram from of a spiked sludge (10 µg/Kg (d.w.)).

To our knowledge, the presence of glucocorticoids in sludge has only been studied in two papers. Liu *et al.* [17] developed a method to determine prednisone, prednisolone, cortisone, cortisol and dexamethasone, but no residue of glucocorticoids was found. Nevertheless, Fan *et al.* [25] determine prednisone, prednisolone, corticosterone, cortisone, cortisol, methylprednisolone and dexamethasone in sludge and found five of them within the range of 0.05 to 0.31 μg/Kg (d.w.).

5 Conclusions

A sensitive analytical method was developed to determine nine glucocorticoids simultaneously in sludge samples with pressurised liquid extraction as an extraction technique. An on-cell clean-up with n-hexane was applied to defat the sludge before the PLE with methanol:acetone (80:20). A SPE clean-up with a polar/non-polar sorbent was applied in order to minimise the matrix interference and reduce the ion suppression. The two clean-up stages ensured that the extracts did not have significant matrix effects (<20%). Recoveries were from 8% to 73% depending on the compound.
Purified extracts were analysed by UHPLC-MS/MS and the method showed LOD around 0.5 μg/Kg (d.w.) for the target analytes. Cortisone (<LOQ), cortisol (5.2-6.1 μg/Kg (d.w.)) and prednisolone (<LOQ-6.0 μg/Kg (d.w.)) were detected in some sludge samples.

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3.1.3. Discussion of results

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Although the results of the experimental part of the studies included in this section have been already discussed in their respective papers, the current section presents and discusses the most important aspects of these.

The most relevant aspect observed during the optimisation of chromatographic separation was that the use of acetonitrile as the organic modifier of mobile phase allowed the separation of the epimers betamethasone and dexamethasone, which only differ in one steric position of a methyl group in their structure. However, acetonitrile made the chromatographic separation difficult in the case of the four most polar compounds studied (prednisone, prednisolone, cortisone and cortisol). Since prednisolone and cortisone have the same mass and specific SRM transition does not exist, their chromatographic separation is also mandatory. To overcome this, the use of a ternary mobile phase composition containing both acetonitrile and methanol was proposed. As mentioned in the first study, the previous published papers only consider one of these two epimers (dexamethasone or betamethasone) and, therefore, the developed method allows the simultaneous determination of nine glucocorticoids including these two epimers in less than 13 min, helped by the use of a short column with sub-2μm particles, which has a higher efficiency than other conventionally columns.

As regards mass spectrometry, extensive optimisation was performed in terms of ionisation performance and fragmentation study because, depending on the ionisation mode (positive or negative), different results in terms of selectivity and sensitivity are obtained for these compounds. Thus, formate adducts which are selected as precursor ions for SRM measurements were the predominant ions obtained under negative ionisation. When positive ionisation was tested, in-source fragmentation was observed due to losses of water molecules and, moreover, formation of sodium adducts was also obtained. These features reduce sensitivity due to the existence of multiple precursor ions and so negative ionisation has proven to be the best option for the determination of glucocorticoids with SRM measurements [1]. Moreover, ESI was compared to APCI but better sensitivity was obtained with ESI. For each compound, three SRM transitions were chosen, the most abundant for quantification and the other two for confirmation purposes, in line with the guidelines of Commission Decision 2002/657/EC [2]. The optimised UHPLC-(ESI)MS/MS method was applied to both studies.

In the optimisation of the extraction process for aqueous samples, three different polymeric sorbents were tested, with both Bond Elut Plexa and Oasis HLB

providing recoveries above 90% and repeatabilities (%RSD, n=3) below 1% for all of the glucocorticoids in spiked ultrapure water. Therefore, Bond Elut Plexa was chosen because less research has been conducted on it. However, when the method was applied to samples, a high matrix effect was observed and so it was decided to dilute the final extract until a matrix effect below 20% was obtained [3].

In the second study, the extraction of glucocorticoids from sewage sludge was attempted. As previously discussed, very few papers report analytical methods for their determination in sludge and it was observed that the recoveries obtained in these papers were around 50% in most cases [4,5]. Moreover, the recoveries obtained during PLE extraction for these compounds are highly dependent on the time between the samples being spiked and extracted. Thus, if samples are rapidly extracted after spiking, the recoveries are close to 100%. However, if longer periods (days) are taken, the recoveries decrease until they stabilise at the results reported in the study, which are more consistent with those reported in some of the studies found in literature.

Because of the semi-quantitative recoveries obtained for some compounds and the high matrix effect occurring in sludge matrices, an on-cell clean-up with n-hexane was proposed to obtain a more selective extraction and improve LODs. Furthermore, the SPE method developed in the first study was carried out after PLE to improve the LODs of the developed method even further.

With both extraction methods developed for aqueous and sewage sludge matrices, LODs at few ng/L and ng/g (d.w.) were obtained, which make them suitable for application to environmental samples. With respect to the samples analysed, eight glucocorticoids included in the study were detected (four of them were determined) in sewage samples and only flumethasone was not detected in any of the analysed samples. The most abundant glucocorticoids were cortisone and hydrocortisone, which were found at concentrations higher than 100 ng/L in both influent and effluent sewage samples from both STPs. Moreover, both betamethasone and dexamethasone were detected (below LOQ) in most of the sewage samples analysed, which confirms the need to separate both isomers chromatographically because they can have different behaviour during sewage treatment and toxicological effects on the surrounding environment. For sewage sludge samples taken from the same STPs, three (prednisolone, cortisone and hydrocortisone) of the four compounds determined in sewage were also detected and two of them were determined at concentrations higher than 5 ng/g (d.w.),

which demonstrates their presence in this kind of matrix. In addition, these compounds are partially removed during sewage treatment but they are still found at lower concentrations in effluent sewage, as can be concluded from the results obtained. However, none of the compounds included in the study were found in the river water samples analysed. These results are in line with previous published papers which indicate that the presence of glucocorticoids in river water samples is negligible because only cortisone and hydrocortisone have been detected in samples from China [5] and Hungary [6] at levels around 2 ng/L.

Lastly, the presence of these compounds has been demonstrated in the samples taken from the aforementioned STPs for which the presence of these compounds had never been reported before.

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3.2. Determination of polyether ionophores in environmental samples

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In this section, the studies focus on the development of sensitive LC-MS/MS methods for the determination of polyether ionophores in river water, sewage and sewage sludge samples. Moreover, a comparison is presented between UHPLC methods coupled to MS/MS and HRMS for the simultaneous determination of polyether ionophores and glucocorticoids in sewage samples.

The development of analytical methods for their determination in environmental samples is necessary because most of the methods previously published only include one or two compounds. Few studies have reported their occurrence in environmental samples [1-7] and even fewer in the case of sewage and sewage sludge samples, for which the information is almost inexistent [3,8].

In line with one of the objectives of this Thesis, both a fused-core amide polar-embedded reversed-phase chromatographic column (RP-amide) and a sub-2 μm C_{18} UHPLC column were tested for the separation of polyether ionophores, because the chromatographic separations previously reported have displayed a number of drawbacks, such as high retention and poor resolution between some compounds. The RP-Amide column was included in the study because this column had not been tested before for the separation of these compounds and very few applications have been reported (none of them for environmental analysis). Thus, the chromatographic method optimisation was included in the first study, which also included the testing of Oasis HLB and Oasis MAX SPE sorbents for extracting polyether ionophores from aqueous samples, because of the acidic properties of these compounds.

The second study focuses on the development of a method for determining polyether ionophores in sewage sludge samples using PLE, because this extraction technique has not been used before for extracting these compounds from sewage sludge samples and it has proven to be very useful for extracting other EOCs [9]. The methods previously reported were based on solid-liquid extraction (shaking) or ultrasonic solvent extraction (USAE) and they have been applied to soils, sediments, manure, litter and sewage sludge for extracting these compounds, but recoveries lower than 10% were reported for sewage sludge. Therefore, PLE was selected as the extraction technique because it can provide extraction rates higher than the aforementioned techniques and it has previously been used to extract monensin and salinomycin from soil samples [10,11]. In addition, in the study presented here, different strategies are tested for reducing the matrix effect that occurs when sludge samples are analysed by LC-MS/MS using an ESI interface, because this is an

important issue in the development of analytical methods for environmental analysis.

As mentioned previously, the last of the presented studies aims to compare tandem mass spectrometry and high resolution mass spectrometry for the determination of veterinary drugs in sewage samples. The study hopes to show the major benefits and drawbacks of each mass spectrometric technique when complex samples are analysed. To this end, an SPE method followed by LC has been developed to determine glucocorticoids and polyether ionophores simultaneously in a single analysis because both groups of compounds are used in veterinary medicine. As mentioned, this study was conducted in collaboration with Dr. Josep Caixach's Group at the Mass Spectrometry Laboratory/Organic Pollutants Department of the Institut de Diagnosi Ambiental i Estudis de l'Aigua/Consell Superior d'Investigacions Científiques (IDAEA-CSIC) in Barcelona.

Lastly, in the first study, river water samples were analysed from the rivers Ter, Llobregat, Ebre and Francolí, and influent and effluent sewage samples were analysed from STPs located in Tarragona and Reus. In the second study, sewage sludge samples were analysed from five STPs located in Tarragona, Reus, Blanes, Castell-Platja d'Aro and Palamós. For all of these STPs, data regarding the occurrence of polyether ionophores had never been reported before. In the third study, sewage samples from the STPs located in Tarragona and Reus were analysed.

The results of these studies have been published in the *Journal of Chromatography A 1263 (2013) 7-13, Journal of Chromatography A 1285 (2013) 31-39* and *Journal of Mass Spectrometry 49 (2014) 585-596*.

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3.2.1. Novel amide polar-embedded reversed-phase column for the fast liquid chromatography-tandem mass spectrometry method to determine polyether ionophores in environmental waters

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NOVEL AMIDE POLAR-EMBEDDED REVERSED-PHASE COLUMN FOR THE FAST LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD TO DETERMINE POLYETHER IONOPHORES IN ENVIRONMENTAL WATERS

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Abstract

A fast chromatographic method has been developed that takes less than 5 min per run to determine five polyether ionophores with a novel amide polar-embedded reversed-phase column coupled to a triple quadrupole mass spectrometer. A comparison between Oasis HLB and Oasis MAX sorbents for the solid-phase extraction was done. Oasis HLB sorbent gave recoveries close to 90% and the repeatability (%RSD, 25-100 ng/L, n=3) of the method was less than 7% for all compounds in all matrices. The presence of polyether ionophores in environmental waters such as river water and sewage was investigated. Monensin and narasin were frequently determined in influent and effluent sewage at concentrations from 10 ng/L to 47 ng/L in influents and from 6 ng/L to 34 ng/L in effluents. In river waters, polyether ionophores were not detected in any sample.

Keywords: *Polyether ionophores; Coccidiostats; Liquid chromatography-tandem mass spectrometry; Solid-phase extraction; Sewage; River water.*

1 Introduction

Polyether ionophores (also known as ionophores) are antibiotics mainly used to treat coccidiosis disease in livestock farms. These compounds are considered as emerging environmental contaminants because their use has been on the rise in many developed countries [1,2]. There is international concern over the potential risk of these compounds to ecosystems as they have antimicrobial potency [2,3]. Ionophores are also used as growth promoters and their usage in the European Union is governed by the Directive 1831/2003/EC [4]. Antibiotics, such as polyether ionophores, are often combined with animal feed for their application. This practice can lead to exposure to veterinary antibiotics in the environment via the excretion of these substances with urine and faeces (e.g. levels around 80% for lasalocid that had been administered to chickens) [5].

Occurrence of ionophores and their behaviour in sewage treatment plants (STPs) has not been subject to thorough research. The presence of these antibiotics into domestic sewage is probably not so apparent as they are only used for veterinary practices. However, discharges from veterinary clinics and runoff from agricultural applications into municipal sewers are potential sources of contamination [6]. To date, only two ionophores (salinomycin and monensin) have been detected in sewage from STPs in Australia and authors reported that their occurrence is much lower than in the surface waters studied, with monensin being detected in 94% of samples compared to 23% in sewage [2]. Also, Zhou et al. have developed a multi-residue method for the determination of 50 antibiotics, including three ionophores (salinomycin, narasin and monensin), in several matrices but they did not find any ionophore residue [7].

It is well known that ionophores can form lipophilic pseudo-macrocyclic neutral complexes with alkali or alkaline earth metals at pH>pK_a [8]. Monensin, salinomycin, narasin, lasalocid and maduramicin have a chemical structure based on various pentacyclic or hexacyclic ethers, a carboxylic acid group and at least one terminal alcohol group. Thus, the alcohol group situated at one end of the molecule bonds with the carboxylic acid group at the other end with a hydrogen bond. This structure wraps around the metal and there is a dipole interaction between the oxygen from the ethers and the metal, such as with a chelator agent. These complexes can cross biological membranes, which is the origin of their biological activity. Lasalocid is based on benzoic acid rather than an aliphatic carboxylic acid and it therefore has a pK_a ~3 while the rest of the ionophores have a pK_a ranging between 4 and 5 (these pK_a were calculated with the SPARC v4.6 on-line calculator [9]).

Analytical strategies for extracting polyether ionophores from aqueous samples involve solid-phase extraction. Studies into to the analysis of waters suggest that polymeric

balanced polar/non-polar sorbents are the most commonly used [2,6,8, 10-14]. Nevertheless, C_{18} has also been used as solid-phase sorbent in the extraction of coccidiostats in river water with recoveries of close to 90% [15]. Strong cation exchange (MCX) and weak cation exchange (WCX) mixed mode sorbents have also both been studied, but the results do not seem promising [14]. Recently, a direct aqueous supercritical fluid extraction (SFE) was developed for the determination of three ionophores in water. However, this extraction technique coupled on-line with LC-MS/MS has not yet been applied to samples [16].

Liquid chromatography is the best option for the determination of ionophores. Stationary reversed phases (normally C_{18}) are used due to the lipophilicity of these compounds [2,10-21]. Nonetheless, polyether ionophores have a great number of functional groups capable of interacting by hydrogen bonds. This fact, combined with their lipophilicity, means that the novel polar-embedded reversed phase columns may be suitable for their chromatographic separation [22]. An amide group is generally used to modify the alkyl chains in the commercial columns, providing an enhancement in the retention factor and selectivity for polar compounds (when they can act as hydrogen bond donors) compared to conventional reversed phase columns [23].

With regard to detection techniques, ultraviolet or fluorescence have been used, but the absence of chromophore groups in these molecules makes the derivatisation step indispensable [24,25]. Therefore, mass spectrometry is the best detection technique for these compounds, as suggested in a large number of papers published about this technique. Ionophores are usually detected in positive mode due to their capacity to form sodium adducts in electrospray ionisation (ESI) [2,10-19,26-31]. Lasalocid was the only ionophore determined in negative mode, despite the presence of a carboxylic acid in its structure [32]. This is probably because of the formation of neutral complexes in the source when the ionophores are deprotonated. In addition, an atmospheric-pressure chemical ionisation (APCI) source was also used in studies by Schlüsener *et al.* [20,21] in order to determine certain antibiotics in soil and liquid manure. The ionophores monensin and salinomycin were investigated but detailed information with respect to the usage of APCI rather than ESI was not recorded. The authors recommend the use of APCI because this method of ionisation is less vulnerable to matrix effects than in the case of ESI [33].

The aim of this paper is the development of a reliable analytical method based on liquid chromatography-(electrospray)tandem mass spectrometry (LC-(ESI)-MS/MS) with a SPE preconcentration step for the determination of the most commonly used polyether ionophores in sewage and river water. A polar-embedded reversed phase column

with fused-core particles was used to obtain a fast chromatographic method, as well as for contributing to broadening knowledge about the chromatogra-phic behaviour of these compounds. In order to evaluate the presence of polyether ionophores, samples from two STPs and four rivers in Catalonia were studied.

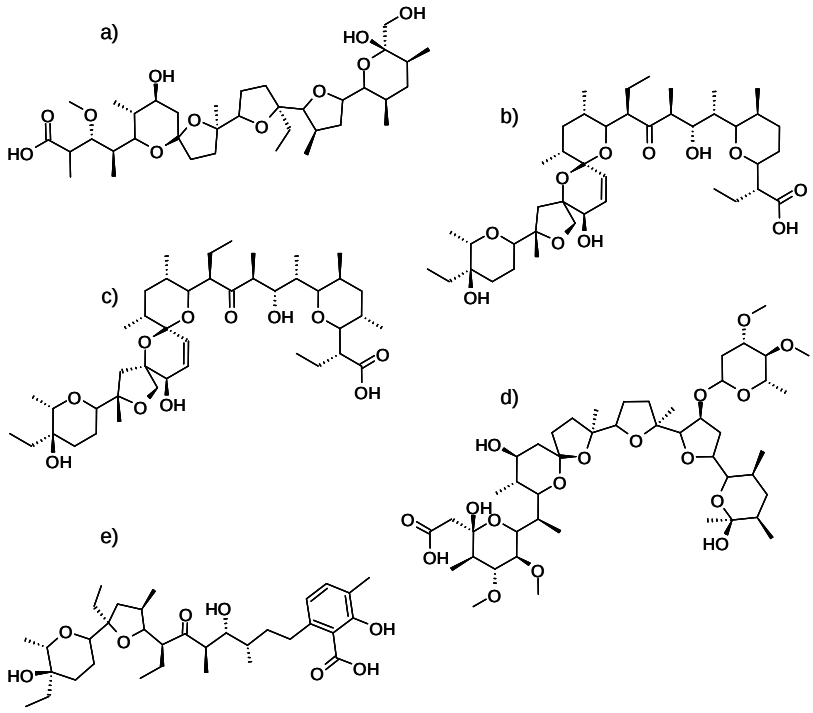

Fig. 1. Chemical structure of polyether ionophores: a) monensin; b) salinomycin; c) narasin; d) maduramicin; e) lasalocid.

2 Experimental

2.1 Reagents and standards

Lasalocid sodium salt solution (100 mg/L in acetonitrile), maduramicin ammonium salt, monensin sodium salt, narasin, salinomycin and the internal standard nigericin sodium salt were purchased from Sigma-Aldrich (St. Louis, USA). Their chemical structures are shown in Figure 1. Stock solutions of individual standards at 1000 mg/L (except for lasalocid) were prepared in methanol and they were stored at -20 ºC. A mixed working solution was prepared daily in water/methanol/acetonitrile (2:1:1) acidified with formic acid at 0.1%.

Ultrapure water was obtained using an ultrapure water purification system

from Veolia waters (Sant Cugat del Vallés, Spain). Acetonitrile (ACN), ethyl acetate (EtOAc) and methanol (MeOH) were of HPLC grade from Prolabo (VWR, Llinars del Vallès, Spain). Formic acid for LC-MS analysis was purchased from Merck (Darmstadt, Germany) and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain).

2.2 Sample preparation

The presence of polyether ionophores in environmental waters such as river water and sewage was investigated in Catalonia (Spain), in particular, in the Tarragona area located in the southeast of the region. River water samples were collected from four Catalan rivers (Ebre, Francolí, Llobregat and Ter). The Ebre is the most plentiful river in Spain and it empties into a delta into the Mediterranean Sea. Water from the Llobregat and Ter Rivers is extensively used for agriculture and industry uses, while the Francolí River passes through several agricultural holdings.

Influent and effluent sewage was collected from two sewage treatment plants (STPs) in the area of Tarragona (Catalonia, Spain). The STPs receive urban sewage and industrial discharges from a population around to 140,000 inhabitants in the case of STP1 and 107,000 in the case of STP2. Both use activated sludge for biological treatment.

All samples were collected using pre-cleaned amber glass bottles and were filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborough, UK). Typically, samples were analysed within three days of their collection and they were stored until analysis in the refrigerator at 4 ºC.

2.3 LC-(ESI)MS/MS analysis

Chromatographic analysis was performed with an Agilent 1200 series Rapid-Resolution liquid chromatograph (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). The chromatographic column used was an Ascentis Express RP-Amide (100 x 2.1 mm, 2.7 μm) from Supelco (Sigma-Aldrich) with fused-core technology. A Zorbax Eclipse XDB-C_{18} (50 x 4.6 mm, 1.8 μm) column from Agilent was also tested in the chromatographic optimisation.

To achieve fast chromatographic analysis, the oven temperature was maintained at 50 ºC in order to reduce the backpressure. Therefore, the flow rate could be increased up to 1 mL/min. The injection volume was 20 μL. A gradient elution was used for the chromatographic separation. Solvent A was ultrapure water with formic acid (0.1%) and solvent B was methanol/acetonitrile (50:50 v/v) with formic acid (0.1%). The gradient was started isocratic at 70% for 3 min and then increased to 85% in 0.5 min and up to 95% in a further 1.5 min. It then remained constant for 1 min and then returned to initial conditions in 0.5 min. All the compounds were eluted in less than 5 min.

Injections without a column of individual standards were used to optimise the conditions of mass spectrometer. These conditions were as follows: nebulizer pressure of 50 psi, drying gas (N_2) flow rate of 12 L/min, drying gas temperature of 350 ºC, and capillary voltage of 3500 V in positive mode. The acquisition mode used was multiple reaction monitoring (MRM) and the cone voltage and collision energies were therefore optimized in order to select three characteristic MRM transitions for each compound, as shown in Table1.

Table 1. LC-MS/MS acquisition parameters in MRM mode.

Compound	t_R (min)	CV[a] (V)	Precursor ion (m/z)	Product ions[b] (m/z)
Monensin	2.2	180	693.4 $[M+Na]^+$	**461.3** (55) $[M+Na-2H_2O-C_{11}H_{16}O_3]^+$
				675.4 (35) $[M+Na-H_2O]^+$
				479.3 (55) $[M+Na-H_2O-C_{11}H_{16}O_3]^+$
Lasalocid	3.2	180	613.4 $[M+Na]^+$	**377.3** (35) $[M+Na-C_{13}H_{16}O_4]^+$
				595.4 (20) $[M+Na-H_2O]^+$
				577.3 (30) $[M+Na-2H_2O]^+$
Maduramicin	3.5	180	939.5 $[M+Na]^+$	**877.5** (35) $[M+Na-H_2O-CO_2]^+$
				895.5 (50) $[M+Na-CO_2]^+$
				859.5 (70) $[M+Na-2H_2O-CO_2]^+$
Salinomycin	3.8	180	773.5 $[M+Na]^+$	**431.2** (55) $[M+Na-C_{19}H_{34}O_5]^+$
				531.3 (45) $[M+Na-C_{13}H_{22}O_4]^+$
				755.5 (35) $[M+Na-H_2O]^+$
Narasin	4.6	180	787.5 $[M+Na]^+$	**431.2** (50) $[M+Na-C_{20}H_{36}O_5]^+$
				531.3 (50) $[M+Na-C_{14}H_{24}O_4]^+$
				769.5 (40) $[M+Na-H_2O]^+$
Nigericin (IS)	4.8	180	747.5 $[M+Na]^+$	**703.5** (55) $[M+Na-CO_2]^+$
				729.5 (35) $[M+Na-H_2O]^+$

[a] CV is cone voltage.
[b] Collision Energies are in brackets (eV).
Quantifier ions are in bold.

2.4 Solid-Phase Extraction

Sample volumes were 250 mL, 500 mL and 1000 mL for STP influent, STP effluent and river samples, respectively. Sodium acetate was added to aqueous samples at a concentration of 500 mg/L (0.5%, w/v), a few minutes before extracting the analytes to increase the formation of pseudo-macrocyclic neutral complexes with sodium. The SPE cartridges tested were Oasis HLB (30 mg and 150 mg) and Oasis MAX (30 mg and 150 mg) both from Waters (Wexford, Ireland). A vacuum pump connected to a manifold (Teknokroma, San Cugat del Vallés,

Spain) was used for the SPE procedure.

The Oasis HLB cartridge was preconditioned with 5 mL of MeOH followed by 5 mL of water prior to loading the samples into the cartridge at a flow-rate of ~10 mL/min. Before the elution step, 5 mL of ultrapure water was passed through sorbent and, afterwards, it was dried under vacuum. The analytes were eluted with 10 mL of MeOH and the eluate was evaporated to dryness under a flow of nitrogen gas. The residue was redissolved in 1 mL of water/MeOH/ACN (2:1:1) with 0.1% of formic acid. The extract was filtered with a 0.22 μm PTFE syringe filter before analysis by LC-MS/MS.

3 Results and discussion

3.1 Mass spectrometry

Polyether ionophores are usually detected in positive mode as sodium adducts $[M+Na]^+$ due to their capacity to complex alkali metals, especially sodium. Initial experiments with the ESI source showed that the predominant precursor ion in the MS spectra was $[M+Na]^+$ for all compounds. Moreover, $[M+NH_4]^+$ and $[M+H-H_2O]^+$ also appeared in MS spectra but their intensities were very low. Sodium precursors were intense but when they were fragmented in the collision cell, the fragments observed were not as intense. This is a common pitfall in the fragmentation of metal adducts. Usually, fragmentation patterns of polyether ionophores are not clear, but losses of water or carbon dioxide are frequent. Other fragments that involve significant mass losses are linked to a gas-phase *β*-cleavage reaction for polyether ionophores that contain a ketone group, as suggested by Martínez-Villalba *et al.* [15].

However, it was possible to work in MRM mode with the fragments observed, as one MRM transition for each compound gave sufficient intensity for the quantification of these analytes and two more MRM transitions could be observed for confirmation. Only monensin had very intense ion ratios (82% for q_1/Q and 80% for q_2/Q). The rest of analytes had ion ratios between 16% and 36% for q_1/Q and between 8% and 13% for q_2/Q.

In order to deal with these issues, negative ionisation was studied. The detection in negative mode as $[M-H]^-$ may be suitable as these compounds include a carboxylic acid. However, it does not seem to be the best option because its ability to form metallic neutral complexes increases when ionophores are deprotonated. However, an equilibrium occurs in the electrospray interface between the complexed form (not detectable) and non-complexed form (detectable as $[M-H]^-$). Thus, it is possible to determine these compounds in negative mode when working with an alkaline mobile phase (ammonium formate buffer (pH 8, 10 mM)). Nevertheless, a good chromatographic method has not been accomplished under these conditions and the response obtained in mass

spectrometry does not improve the response of the sodium adducts. In addition, an APCI source was also checked. The ionisation patterns were identical to those obtained with the ESI source in both modes ($[M+Na]^+$ in positive mode and $[M-H]^-$ in negative mode), but the responses were much lower than in ESI and, therefore, the APCI source was discarded as an option. However, Schlüsener *et al.* worked with an APCI source in positive mode and the precursor ions obtained were $[M+NH_4]^+$ because their mobile phases contained ammonium acetate to suppress sodium adducts [20,21].

Based on these results, ESI positive ionisation (as sodium adducts) was selected as the best option for polyether ionophores. Consequently, cone voltages and collision energies were optimised for each compound under this ionisation mode and optimised values are showed in Table 1. The other parameters of ESI source are in Section 2.3.

It is well known that the formation of adducts in the electrospray ionisation may not be very reproducible (increased when environmental samples are analysed) and, therefore, usage of internal standard is recommended [8]. For this purpose, nigericin is preferred by researchers because its structure is very similar to the compounds studied and it was used in this study.

3.2 Liquid chromatography

As mentioned in the introduction, polyether ionophores are usually separated with octadecylsilyl reversed-phase columns. However, polar-embedded reversed-phase columns may offer new potential with respect to the separation of these compounds. Therefore, these two stationary phases were tested.

Firstly, a chromatographic method was developed with a Zorbax Eclipse XDB-C_{18} (50 x 4.6 mm, 1.8 μm) column. In line with previous papers [8], ultrapure water and ACN, both with formic acid (0.1%), were used as mobile phases. The usage of columns with sub-2 μm particle size in rapid resolution chromatographs (600 bar) is strongly linked to applying temperature to reduce the system backpressure. Therefore, the column oven was set at 50 ºC. The complete resolution between all compounds was achieved under optimised conditions in less than 10 min at a flow rate of 1 mL/min. The elution profile of the compounds was as follows: lasalocid, monensin, salinomycin, narasin, maduramicin and nigericin. This method was then transferred to an Ascentis Express RP-amide (100 x 2.1 mm, 2.7 μm) column. Retention factors for both columns were similar, but there were significant differences in selectivity. Therefore, the elution profile was very different in both columns. For example, lasalocid was the least retained compound in C_{18} column, while in the RP-amide column, it was the third compound eluted.

Separation of the compounds was optimised with the RP-amide column under the conditions mentioned, but total resolution between compounds

was not accomplished. Subsequently, MeOH was used as the organic mobile phase instead of ACN. The selectivity changed again and a complete resolution between compounds was possible. However, retention times of compounds were highly increased when MeOH was used due to the relative hydrophobicity of polyether ionophores. In addition, flow rate was limited because the system backpressure was also increased with this mobile phase. In order to obtain a fast chromatographic method, a binary organic mixture with MeOH and ACN (50:50) was tested. The good resolution achieved with MeOH and a high solvent strength and low backpressure provided with ACN led us to believe that it is a promising option. The method was further optimised with this mobile phase and, finally, a chromatographic separation in less than 5 min was achieved with a good resolution between all compounds. The optimised elution gradient is described in Section 2.3 and retention times of compounds are presented in Table 1.

In addition, an alkaline mobile phase (ammonium formate buffer (pH 8, 10 mM) was also tested for the detection of compounds in (-)ESI. However, retention factors were low and good chromatographic separation was not achieved under these conditions.

Several injection volumes and solvents were tested in order to achieve the optimal peak shape and response. In our research, H_2O/MeOH/ACN (50:25:25) with 0.1% of formic acid gave the best response and a volume up to 20 μL did not affect to peak broadening.

3.2 Solid-phase extraction

Depending on the compounds of interest (i.e. whether they have an ionisable group or not), cleaner extracts of sample may be achieved with mixed-mode sorbents (e.g. Oasis MAX) in comparison to non-functionalised polymeric sorbents (e.g. Oasis HLB) because a washing step with organic solvent can be performed. Therefore, this washing step can lead to a reduction in the matrix effect in the extracts. Oasis HLB is the most commonly used SPE sorbent for the extraction of polyether ionophores from aqueous matrices [8]. Nonetheless, the acidic properties of these compounds may be suitable for extracting with a mixed-mode strong anion exchange sorbent (SAX). Oasis MAX has the same polymeric sorbent as Oasis HLB and it has a functional group that is capable of interacting with anionic analytes. Therefore, both SPE sorbents, HLB and MAX, were compared.

Common SPE protocols (specified by the supplier) were tested for HLB (30 mg) and MAX (30 mg) with standard solutions (ultrapure water spiked at 20 μg/L) in order to evaluate their suitability. For HLB: the sample (5 mL) was acidified at pH 3 with formic acid, the washing step was performed with 1 mL of ultrapure water and the compounds were eluted with 1 mL of MeOH. For MAX: the pH of sample (5 mL) was 9

and it was adjusted with ammonium hydroxide, the washing step was performed in two steps (1 mL of ultrapure water (5% ammonium hydroxide) followed by 1 mL of MeOH (5% ammonium hydroxide)) and the elution was carried out with 1 mL of MeOH (5% formic acid). The results obtained with MAX were not as positive as those obtained with HLB because only lasalocid interacts strongly by ion exchange and the rest of analytes (maduramicin, narasin, salinomycin and monensin) were eluted in the washing step with MeOH (5% ammonium hydroxide). Lasalocid was eluted with MeOH (5% formic acid) and this is probably because it includes a benzoic acid in its structure rather than an aliphatic carboxylic acid. Therefore, we left out the washing step with MeOH (5% ammonium hydroxide) in MAX and the elution was performed in a single step with MeOH (5% formic acid) in order to study if MAX provided an enhancement in the retention of these compounds in comparison to HLB.

Subsequently, the recoveries for both sorbents (30 mg), which are shown in Table 2, were evaluated using the conditions stated above. Recoveries for lasalocid and monensin were increased from 76% and 74% with HLB to 90% and 103% with MAX, respectively. Maduramicin, salinomycin and narasin had recoveries around 65% with both sorbents. Thus, it can be seen that lasalocid and monensin interact with MAX's functionalised group, giving better recoveries than HLB.

To improve the efficiency of extraction, Cha *et al.* [11] suggest the addition of sodium chloride to samples (0.005% to 1%, w/v) in order to generate sodium neutral complexes. In our case, sodium acetate (0.5%, w/v; pH 8) was used to deprotonate the ionophores and then to increase the formation of neutral species.

The procedures for HLB and MAX were transferred to 150 mg cartridges and sample volumes were increased up to 100 mL with ultrapure water spiked at 1 μg/L to test different elution solvents (MeOH and EtOAc by HLB, and MeOH (5% formic acid) by MAX) and volumes (5 mL and 10 mL for each one). Recoveries obtained with 10 mL of MeOH by HLB and 10 mL of MeOH (5% formic acid) by MAX as elution solvents are shown in Table 2.

The results are reasonably positive, with recoveries from 88% to 110% with HLB. In contrast, the method is not appropriate with MAX cartridges because recoveries were lower (38% to 63%) and narasin and salinomycin were not retained under these conditions. With regard to solvent elution and its volume, the best results were obtained with 10 mL of MeOH because ethyl acetate is not capable of eluting lasalocid. Moreover, in the case of the other compounds, the recoveries are slightly lower with EtOAc.

Therefore, the addition of sodium acetate tosamples, Oasis HLB (150 mg) as sorbent and a volume of 10 mL of MeOH for the elution were chosen as the optimal conditions.

Table 2. Recoveries by different SPE optimisation experiments.

Compound	Standard procedure (30 mg)		Modified procedure (150 mg)	
	HLB	MAX	HLB	MAX
Monensin	74	103	88	38
Lasalocid	76	90	90	63
Maduramicin	71	64	108	51
Salinomycin	63	71	110	-
Narasin	65	68	108	-

For experimental conditions see text.
%RSD(n=3)<10%.

One litre of ultrapure water spiked at 100 ng/L was passed through to the cartridge and similar recoveries were obtained.

Recoveries and matrix effects were evaluated in three kinds of environmental aqueous samples (river, influents and effluents). In particular, 1 L for river and 500 mL for effluent and 250 mL for influent were tested based on our previous results and experience. The resulting extracts were evaporated to dryness under a flow of N_2 and were reconstituted to 1 mL with water/MeOH/ACN (2:1:1) with 0.1% of formic acid. Recoveries were calculated by spiking samples at 100 ng/L, 200 ng/L and 400 ng/L, respectively, and recoveries ranged from 79% to 123% depending on the analyte and the matrix. Therefore, the procedure with these volumes was chosen as optimal because breakthrough did not occur. The matrix effect was calculated by spiking blank extracts of three matrices at the same concentration as before.

The responses were compared with a standard at same concentration ($(A_{sample}-A_{standard})\bullet 100/A_{standard}$) and results were reasonably good for all analytes, except for salinomycin, which underwent the strongest matrix enhancement. For monensin, lasalocid, maduramicin and narasin, the matrix effect ranged from -21% to 48% while in the case of salinomycin, it ranged from 94% to 161%. Therefore, a matrix-matched calibration curve was adopted in order to obtain the most accurate results possible.

3.4 Method validation

For the validation of the method developed, linear range, method limits of quantification and detection, repeatability (expressed as relative standard deviation (%RSD)) and recoveries for selected polyether ionophores were evaluated with three kinds of environmental aqueous samples. The linear range of each compound was evaluated by spiking samples at different levels between 0.5 ng/L and 500 ng/L. The method was linear in all ranges between LOQ and 500 ng/L for all compounds and matrices. LOQs were the lowest

point of the calibration curve and LODs were evaluated by spiking blank extracts in order to obtain up to three times the signal/noise ratio. These limits are shown in Table 3. In all cases, a blank sample was also analysed to evaluate the presence of compounds. However, no signal from analytes was observed. Thus, LOQs ranged from 5 ng/L to 20 ng/L and LODs ranged from 0.5 ng/L to 2 ng/L depending of the compound and matrix studied. These limits are slightly better than those reported in the literature, which reports LODs between 1 ng/L and 40 ng/L for surface waters [10,11,13,15,31]. To the best of our knowledge, no data has reported in literature for sewage influent or effluent.

Table 3. Validation values for the SPE-LC-MS/MS method.

Compound	LOD (ng/L)	LOQ (ng/L)	Recovery (%, n=3)	Repeatability (%RSD, n=3)
Monensin	0.5[a]	1	91	1
	1[b]	2	91	0.3
	2[c]	4	95	3
Lasalocid	0.5	1	97	0.9
	1	2	88	1.8
	2	4	86	7
Maduramicin	1	5	96	1
	5	10	100	6
	10	20	96	6
Salinomycin	0.5	1	95	2
	1	2	93	5
	2	4	97	6
Narasin	0.5	1	89	2
	1	2	87	2
	2	4	93	5

[a] River water; Recovery and %RSD calculated at 25 ng/L.
[b] Effluent sewage; Recovery and %RSD calculated at 50 ng/L.
[c] Influent sewage; Recovery and %RSD calculated at 100 ng/L.

Recoveries were obtained from spiked samples (25 ng/L for river, 50 ng/L for effluent sewage and 100 ng/L for influent sewage). Analysis was carried out by triplicate to calculate the repeatability (%RSD, n=3). Results for recoveries and repeatability are shown in Table 3. Recoveries of polyether ionophores ranged from 89% to 97% for river, from 87% to 100% for effluent sewage and from 86% to 97% for influent sewage. Repeatability was less than 7% in all cases. These results show the benefits of the method developed and its potential for application to environmental water samples.

4 Application to environmental samples

Several samples from river water (4), influent (6) and effluent (6) sewage were analysed in order to observe the applicability of the method. Table 4 shows the results and ion ratios in line with the Commission Decision 2002/657/EC [34] for analysed samples, with qualifier ion ratios in spiked samples shown in bold. Analysis of river water shows little evidence of the presence of polyether ionophores in these samples. The MRM quantification chromatograms showed a slight response (at the retention time of compounds) for monensin, lasalocid and narasin in some samples, but their presence cannot be confirmed because the MRM confirmatory transitions did not correspond with the ion ratio expected for these compounds.

Table 4. Concentrations (ng/L) and qualifier ion ratios of polyether ionophores in studied samples.

Sample	Monensin		Salinomycin		Narasin	
Influent		**(78,84)**		**(36,8)**		(37,10)
I1	15.9	(76,81)			46.8	(34,14)
I2	16.4	(74,96)			47.0	(36,15)
I3	12.3	(77,99)	32.5	(35,15)	42.6	(44,12)
I4	13.5	(74,98)			43.1	(36,13)
I5	10.2	(70,99)			<LOQ	
Effluent		**(80,83)**		**(38,7)**		**(40,11)**
E1	23.9	(71,87)			10.2	(39,14)
E2	32.0	(71,88)			13.1	(37,10)
E3	22.1	(83,82)			9.6	(30,11)
E4	34.0	(78,82)			8.8	(41,14)
E5	23.5	(77,80)			6.4	(33,15)

Qualifier ion ratios (%) in spiked samples are in bold.
Qualifier ion ratios (%) in samples are in brackets.

Monensin and narasin had previously been determined in surface waters at levels between 3 ng/L and 38 ng/L by various authors [10,11,13]. Salinomycin has also been determined between 4 ng/L and 7 ng/L [10]. To the best of our knowledge, information on the presence of lasalocid in river water has not been reported.

With regard to sewage, monensin and narasin were determined in 5 samples of influents at levels between 10 ng/L and 16 ng/L and 43 ng/L 47 ng/L, respectively, and salinomycin was determined in one sample at 32 ng/L. Moreover, a signal at the retention time for lasalocid was observed in the MRM chromatogram in several samples. However, their qualifier ion ratio for the MRM transition (613 > 595) was higher than the expected

ratio. Therefore, the presence of lasalocid in the influent sewage analysed cannot be confirmed.

In the case of effluents, only monensin and narasin were determined in 5 samples at levels between 22 ng/L and 34 ng/L for monensin and 6 ng/L and 13 ng/L for narasin. Levels found for monensin indicate that it had not been eliminated in sewage treatment plants. However, the concentrations of narasin were lower than in influents. MRM chromatograms for influent and effluent samples are shown in Figure 2. Only Watkinson *et al.* [2] have determined polyether ionophores in sewage, reporting concentrations for salinomycin of up to 300 ng/L in influents (only 5% of samples are positive) and 20 ng/L for monensin in effluents (26%).

The results indicate that the presence of these compounds in sewage is significant as they are used exclusively in veterinary and their occurrence into sewage of urban areas is not so obvious. Nevertheless, studies indicate that polyether ionophores are the most commonly used antibiotics in agricultural industries. In the USA and New Zealand, around 50% (by weight) of the total amount of antibiotics used are ionophores [3]. In the EU, monensin and salinomycin are widely used as growth promoters. In other countries, narasin and lasalocid have also become widespread for this use [3]. Therefore, the introduction of these compounds into sewage treatment plants may be due to their extensive use in livestock farms.

5 Conclusions

The novel amide polar-embedded reversed-phase column was successfully applied for the separation of polyether ionophores. Moreover, the fused-core technology combined with the increase of temperature provided a rapid chromatographic method, taking less than 5 minutes, with a good resolution for all compounds. These compounds do not have great hydrophilic affinity. However, selectivity is changed in comparison to the usual C_{18} stationary phase because polar groups of compounds interact with the amide group.

Recoveries in the SPE procedure for ionophores are close to 90% or above when sodium acetate is added to water samples. Repeatability (%RSD, n=3) is less than 7% and LODs and LOQs for these compounds were the lowest reported in the analysis of environmental waters.

The method was successfully applied to determine five polyether ionophores in several river water and sewage samples taken in Catalonia. The most commonly detected compounds were monensin and narasin in influents and effluents sewage.

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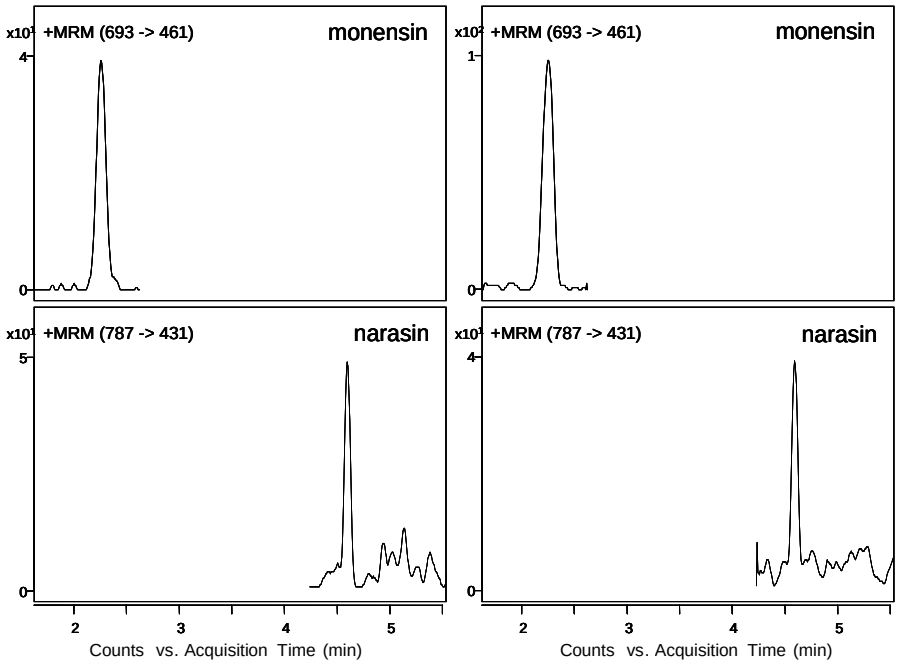

Fig. 2. MRM chromatograms obtained of an influent (left) and effluent (right) sewage collected in STP 2.

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ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
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3.2.2. Determination of polyether ionophores in urban sewage sludge by pressurised liquid extraction and liquid chromatography-tandem mass spectrometry. Study of different clean-up strategies

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DETERMINATION OF POLYETHER IONOPHORES IN URBAN SEWAGE SLUDGE BY PRESSURISED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. STUDY OF DIFFERENT CLEAN-UP STRATEGIES

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Abstract

A method for the determination of five polyether ionophores in urban sewage sludge has been developed. The extraction of compounds was performed by pressurised liquid extraction using acetone, while florisil was used for in-cell clean-up to minimise the matrix effect in the sludge extracts. An amide polar-embedded reversed-phase column was used for the chromatographic separation with a rapid-resolution liquid chromatograph coupled to a tandem mass spectrometer. Moreover, several clean-up strategies such as in-cell and on-cell clean-up and solid-phase extraction clean-up, among others, were tested and their results are discussed in the present paper. Recoveries (10 and 250 μg/Kg in dry weight (d.w.), n=6) were close to 90%, repeatability and reproducibility (%RSD, 10 and 250 μg/Kg (d.w.), n=6) were less than 10% and12%, respectively. Limits of detection (LODs) and limits of quantification (LOQs) ranged between 0.5 and 1 μg/Kg (d.w.) and between 1 and 5 μg/Kg (d.w.), respectively. The method was applied to samples collected in five sewage treatment plants in Catalonia. Monensin and narasin were detrmined in some sludge samples at concentrations from <LOQ to 3.5 μg/Kg (d.w.) for monensin and from <LOQ to 3.7 μg/Kg (d.w.) for narasin.

Keywords: *Polyether ionophores; matrix effect; liquid chromatography-tandem mass spectrometry; pressurised liquid extraction; in-cell clean-up; sewage sludge.*

1 Introduction

A broad spectrum of organic chemicals is essential to modern society. Once discharged from industrial, domestic and urban sources into the sewer system, lots of organic chemicals may be transferred into the residual solids during sewage treatment. Recycling treated sewage sludge on land is internationally recognised as the most sustainable option for managing the residual sludge from urban sewage treatment plants (STPs) and most risk assessments demonstrate that this practice does not place human health at risk from the majority of organic contaminants studied worldwide. Nonetheless, continued studies are necessary to identify new organic contaminants in order to ensure the long-term sustainability of the agricultural route for treated sludge management [1].

Polyether ionophore antibiotics are used to treat infections in poultry and livestock farms. They are also used as growth promoters for ruminants [2]. Their usage in the European Union is governed by the Regulation 1831/2003/EC [3]. Antibiotics can cause resistance among natural bacterial populations and they have high toxicity to bacteria at low concentrations. Hence, they are classified as a priority substance risk group in the environment [4].

The presence of polyether ionophores in surface waters [2,4-13] or soils [6,14-16] and sediments [5,9,13] is a well known issue. These compounds can reach lands or surface waters via runoff from livestock farms or by leaching of animal faeces, slurry or manure [17]. However, polyether ionophores have also been found in urban sewage [4,12,13], probably as a result of discharges from veterinary clinics into municipal sewers [2] or from waste from meat processing plants and abattoirs, as suggested by Watkinson *et al.* [4].

To the best of our knowledge, the occurrence of polyether ionophores has only been studied in sewage by Watkinson *et al.* [4] in Australia, Zhou *et al.* [13] in China and our group [12] in Spain. To date, only salinomycin and monensin have been determined in Australian sewage [4] and salinomycin, monensin and narasin in Spanish sewage [12] at concentrations of around a few ng/L, both in the case of influents and effluents. In the study in China, no residue of polyether ionophores was found in sewage [13]. The presence of these compounds in sewage led us to think that sewage sludge could be a possible destination of polyether ionophores due to their lipophilic character. Their occurrence in sewage sludge had not received the same attention as other antibiotics because polyether ionophores are exclusively used for veterinary applications and their presence in sewage had not been expected.

Analytical methodologies for extracting polyether ionophores from solid matrices are usually based on conventional solid-liquid extraction (shaking) or ultrasonic solvent extraction (USE), and were applied to

soils [5,6,16], sediments [9,13], manure [13,18], litter [16] and sludge [13]. However, pressurised liquid extraction (PLE) has become a versatile tool for the extraction of a wide range of organic compounds in environmental solid matrices with the semi-automation of the extraction process and lower time and solvent consumption than other methodologies mentioned above. Hence, PLE was applied to extract monensin and salinomycin from soil [14,15]. In addition, most of these methodologies were followed by solid-phase enrichment and/or a clean-up stage.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most commonly used technique for the determination of polyether ionophores. Usually, octadecylsilyl reversed phases are used due to the lipophilicity of these compounds [17]. However, polar-embedded reversed phase columns can be used because polyether ionophores have a great number of functional groups capable of hydrogen bond interactions and, as a result, a different selectivity can be achieved [12,19,20].

A large number of papers using tandem mass spectrometry (MS/MS) as a detection technique for these compounds have been published [4,5,7-12,16,21-29]. However, UV-Vis or fluorescence detection have also been used [30,31]. Polyether ionophores are usually detected in positive mode due to their capacity to form sodium adducts in electrospray ionisation (ESI) but atmospheric-pressure chemical ionization (APCI) was also used in studies by Schlüsener *et al.* [14,15,18].

The main objective of the present study is to develop an analytical method for the determination of five polyether ionophores in sewage sludge by pressurised liquid extraction (PLE) and liquid chromatography-(electrospray)tandem mass spectrometry (LC-(ESI)-MS/MS). To this end, an evaluation was attempted of the occurrence of these compounds in sewage sludge, as they had previously been determined in sewage from the STPs studied in Catalonia [12].

2 Experimental

2.1 Reagents and standards

Lasalocid sodium salt solution (100 mg/L in acetonitrile), maduramicin ammonium salt, monensin sodium salt, narasin and salinomycin were purchased from Sigma-Aldrich (St. Louis, USA). Chemical structures of compounds are shown in Figure 1. Stock solutions of individual standards at 1,000 mg/L (except for lasalocid) were prepared in methanol and they were stored at -20 ºC. A mixed working solution (10 mg/L) was prepared weekly in methanol and standard solution with water/methanol/acetonitrile (2:1:1) with formic acid (0.1%) was prepared daily.

Ultrapure water was obtained using an ultrapure water purification system provided by Veolia Water (Sant Cugat del Vallès, Spain). Acetone,

acetonitrile (ACN), dichloromethane (DCM), ethyl acetate (EtOAc), isooctane, methanol (MeOH), methyl tert-butyl ether (MTBE) and n-hexane were of HPLC grade and supplied by Prolabo (VWR, Llinars del Vallès, Spain). Formic acid (FA) for LC-MS analysis was purchased from Merck (Darmstadt, Germany) and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain). The alumina, diatomaceous earths, florisil and silica used in PLE were purchased from Sigma-Aldrich.

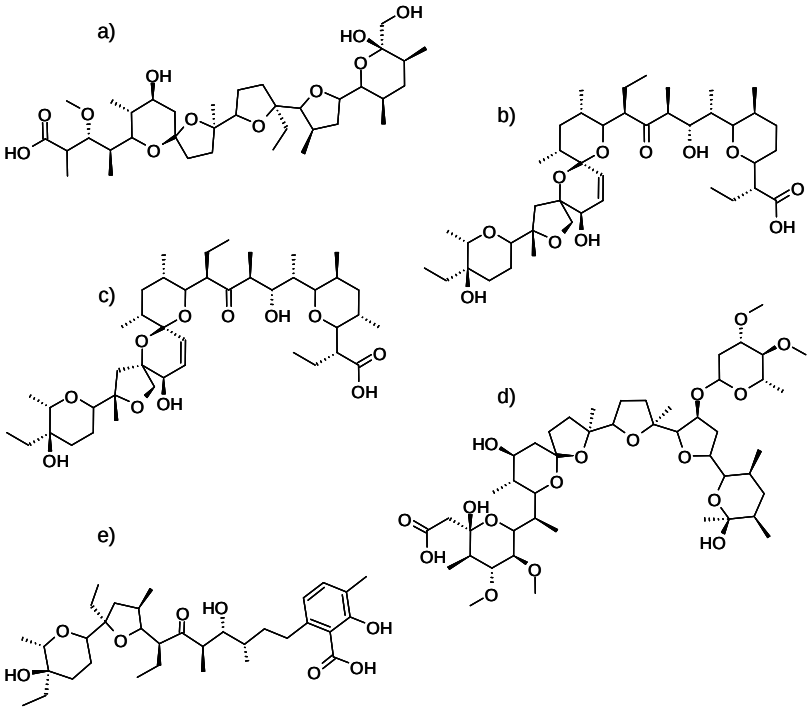

Fig. 1. Chemical structure of polyether ionophores: a) monensin; b) salinomycin; c) narasin; d) maduramicin; e) lasalocid.

2.2 Sampling

The sewage sludge samples were collected using pre-cleaned amber glass flasks from five sewage treatment plants (STPs) located in two different regions of Catalonia (Spain). Two of these STPs were in the Tarragona region (Tarragona STP and Reus STP) and the other three were in the region of Girona (Blanes STP, Palamós STP and Castell-Platja d'Aro STP). These sewage treatment plants have a secondary treatment based on activated sludge biological treatment, while the STPs in the Girona region have an additional tertiary treatment. All plants are located in cities with a population greater than 100,000 inhabitants and

receive urban wastewaters and some industrial and agricultural discharges. The average flow rates were between 23,500 and 35,000 m^3/day and the biological oxygen demands (BOD_5) ranged from 300 to 470 mg/L for all of the sewage treatment plants.

2.3 Pressurised Liquid Extraction

Sludge was frozen before being lyophilised and then crushed in a mortar and pestle and sieved (125 μm) to obtain particles with the same diameter. Spiked samples were prepared by adding the stock mixture of standards in acetone (the required volume to wet and cover the sludge). The solvent was slowly evaporated at room temperature inside an extractor hood with frequent homogenisation of the sample.

A glass fibre filter was placed at bottom of the 11 mL stainless steel extraction cell. It was then filled with 1 g of diatomaceous earth, 1 g of florisil, 1 g of freeze-dried sample mixed with 1 g of florisil and 1 g of diatomaceous earth and finally, with another gram of diatomaceous earth. A glass fibre filter was placed at top of the cell and then, it was compacted and closed before extraction. The extraction was carried out with one cycle of acetone at 80 ºC and 1,500 psi during 10 min. The preheating time was 5 min, flush volume was 30% of cell volume and purge time was 120 s.

The sample extract was evaporated to dryness under a flow of nitrogen gas and it was redissolved in 10 mL with a mixture of water/MeOH/ACN (2:1:1) with 0.1% of formic acid. The extract was filtered with a 0.22 μm PTFE syringe filter prior to being analysed by LC-MS/MS.

2.4 LC-(ESI)MS/MS analysis

Chromatographic analysis was performed with an Agilent 1200 series rapid-resolution liquid chromatograph (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). The chromatographic method and mass spectrometric parameters were adapted from a previous paper [12].

The chromatographic column used was an Ascentis Express RP-Amide (100 x 2.1 mm, 2.7 μm) from Supelco (Sigma-Aldrich) with fused-core technology. The oven temperature was set at 50 ºC and the flow rate was 1 mL/min. The injection volume was 20 μL. The chromatographic separation was performed in gradient elution with ultrapure water (A) with formic acid (0.1%) and methanol/acetonitrile (50:50, v/v) (B) with formic acid (0.1%) as the mobile phases. The elution gradient started at 50% of B, and it was maintained isocratically for 0.5 min. It was then increased up to 70% in 0.5 min and this composition was kept for 4 min. Finally, the gradient was increased up to 95% in 3 min and it was held at this level for 3 min more before returning to initial conditions in 1 min. All of the compounds were eluted in less than 7.5 min.

Mass spectrometric detection was

performed by multiple reaction monitoring (MRM) in positive ESI mode and three transitions for each compound (Table 1) were acquired in accordance with the guidelines established in the Commission Decision 2002/657/EC [32]. Nebuliser pressure (50 psi), drying gas flow rate (12 L/min), drying gas temperature (350 ºC), capillary voltage (+3.5 kV) and cone voltage (180 V) were optimised by the flow injection anaylsis (FIA) of individuals standards. The collision energies corresponding to each transition are shown in Table 1 and the proposed structure of daughters are described in a previous paper [12].

Table 1. LC-MS/MS acquisition parameters in MRM mode.

Compound	t_R (min)	CV[a] (V)	MRM transitions[b] (m/z)		Ion ratio[c] (%)	
					q_1/Q	q_2/Q
Monensin	4.4	180	**693.4**	**461.3** (55); 675.4 (35); 479.3 (55)	98	81
Lasalocid	5.3	180	**613.4**	**377.3** (35); 595.4 (20); 577.3 (30)	27	16
Maduramicin	6.0	180	**939.5**	**877.5** (35); 895.5 (50); 859.5 (70)	14	8
Salinomycin	6.2	180	**773.5**	**431.2** (55); 413.2 (45); 755.5 (35)	19	16
Narasin	7.2	180	**787.5**	**431.2** (50); 531.3 (50); 769.5 (40)	70	46

MRM transitions used for quantification are in bold.
[a] CV is cone voltage.
[b] Collision Energies are in brackets (eV).
[c] Determined by a sludge samples spiked at 250 μg/Kg (d.w.).

3 Results and discussion

3.1 LC-(ESI)MS/MS

The initial chromatographic method was the one developed in a previous paper in which five polyether ionophores were determined in water samples from river and sewage [12]. Because of the higher complexity of sludge samples, it was necessary to modify the chromatographic separation in order to reduce the matrix effect caused by co-eluting compounds of the sample as will be discussed in Section 3.3.

As regards mass spectrometry, polyether ionophores interact with alkali metals generating sodium adducts $[M+Na]^+$ in the electrospray interface. The common fragmentation pathways are losses of water or carbon dioxide. However, fragments that involve significant mass losses are linked to a gas-phase *β*-cleavage [11] but these reactions are not very clear.

3.2 Sample extraction (PLE)

An efficient extraction of analytes from a solid sample (e.g. lyophilised sludge) with pressurised liquid extraction involves the optimisation of some parameters. The solvent is the most important factor, and

temperature, time and number of cycles also have a considerable impact on extraction efficiency. Different pressures, flush volumes and purge times can be tested but significant differences in the extraction are not expected.

Methanol [15,16], water [6] or buffered aqueous solutions on their own [5,9] or with ethyl acetate [33] were used for the extraction of some polyether ionophores from soils and sediments.

Table 2. Recoveries (%) in PLE optimisation.

	Monensin	Maduramicin	Salinomycin	Lasalocid	Narasin
Extraction solvent[a]					
DCM	27	30	35	47	59
EtOAc	22	38	27	50	46
acetone	97	92	96	106	96
MeOH	110	100	111	109	104
Extraction temperature[b]					
40ºC	96	86	83	95	71
60ºC	88	95	84	94	79
80ºC	97	92	96	106	96
100ºC	84	77	77	84	81
Static time[c]					
5 min	97	100	100	94	87
10 min	97	92	96	106	96
15 min	90	95	102	83	87
20 min	95	95	107	82	89

[a]Sludge spiked at 500 µg/Kg d.w.; 80ºC;10 min.
[b]Sludge spiked at 500 µg/Kg d.w.; acetone;10 min.
[c]Sludge spiked at 500 µg/Kg d.w.; acetone; 80ºC.
%RSD(n=3)<10%.

Zhou *et al.* [13] suggest the use of acetonitrile citric buffer to extract them from different environmental solid matrices, including sludge, manure and sediment but only provides efficient recoveries for sediment [13]. Based on papers published and our experience, methanol, acetone, ethyl acetate and dichloromethane were chosen as proper solvents for the extraction of polyether ionophores from lyophilised sludge samples.

One gram of spiked sample (500 µg/Kg d.w.) was mixed with another gram of diatomaceous earth and it was placed into the stainless-steel cell. The initial conditions selected were 80 ºC, 5 min of preheating cell, no heating time and 10 min of static

time. The others parameters are described in the experimental section. The organic sample extracts were evaporated to dryness under nitrogen flow and were redissolved with 2 mL of water/MeOH/ACN (2:1:1) with 0.1% FA. In order to calculate the recoveries, a non-spiked sample was extracted under same conditions and was spiked with analytes at same concentration before the evaporation step. The areas obtained for both experiments were compared in order to calculate the recovery (%). In addition, a non-spiked sample was analysed to check the presence of these compounds in the sludge and they were not found. Results showed (Table 2) that methanol and acetone were very efficient for the extraction (recoveries were close to 100% for all compounds). In contrast, non-polar solvents were not capable of extracting the compounds well (recoveries less than 60%). In addition, the area obtained from post-spiked sample was compared with a standard solution to evaluate the matrix effect (%) obtained with each solvent ($(A_{sample}-A_{standard})\bullet 100/A_{standard}$). The matrix effect was similar for all solvents and ranged between -93% and -23% depending on the compound. Hence, acetone was chosen for the subsequent optimisation experiments because the recoveries were very high, it involves a similar matrix effect to other solvents and it is easier to evaporate than methanol.

The extraction temperature was tested at 40, 60, 80 and 100 ºC with the same conditions stated above. The results showed (Table 2) that the temperature does not have a strong impact on extraction efficiency for all the compounds, except narasin, the least polar compound. The recoveries slightly decreased at 100 ºC. Thus, 80 ºC was selected as the optimal value for the extraction process due to the recoveries obtained for narasin. Moreover, a reduction of the matrix effect was not observed at lowest temperatures.

Static times of 5, 10, 15 and 20 min and up to 3 cycles (of 5 min) were tested to complete the extraction optimisation. The experiments show (Table 2) that static time does not have a significant impact on the extraction of compounds from sludge because in all cases, the recoveries are close to or higher than 90%. However, 10 min was chosen because the recoveries for lasalocid and narasin were slightly higher than other static times tested. With respect to extraction cycles, improvement in the recoveries with two or three cycles was not significant. Therefore, one cycle was chosen for the extraction.

3.3 Sample clean-up strategies

Due to the strong matrix effect observed during the PLE optimisation, which involves ion suppression or enhancement in the LC-(ESI)MS/MS determination, attempts were made to reduce it in order to achieve better quantification of the target analytes. It is therefore an issue that must be solved or, at least, improved in order to achieve a

reliable analytical method. For this reason, our study involves several strategies to deal with this aspect, such as in-cell and on-cell clean-up, solid-phase extraction (SPE) clean-up, improvement of the chromatographic separation and dilution of the extract.
Therefore, this section is divided into four sub-sections depending on the method stages involved.

3.3.1 PLE clean-up

Pressurised liquid extraction clean-up was performed in the cell in two different ways: in-cell clean-up with an adsorbent which retains the interfering substances or on-cell clean-up based on sequential extraction with different kinds of solvent (different polarities) which extracts the interfering substances prior to rejecting them. The operational conditions of PLE were described in Section 2.3 and a spiked sample (500 μg/Kg d.w.) without any clean-up strategy was initially analysed in order to compare the efficiency of the clean-up methods tested. All of the samples were evaporated to dryness and redissolved to 2 mL. The results are shown in Table 3 as apparent recoveries (%) taking into account the recovery yield and matrix effect together [34].

Table 3. Apparent recoveries (%) for PLE clean-up.

	Monensin	**Maduramicin**	**Salinomycin**	**Lasalocid**	**Narasin**
Without (DE)[a]	17	96	23	<10	<10
In-cell					
Florisil	32	113	48	<10	<10
Alumina	12	22	<10	-	<10
Silica	17	81	15	<10	<10
On-cell					
DCM	<10	<10	<10	<10	<10
EtOAc	<10	<10	17	<10	<10
Hexane	15	11	33	<10	<10
MTBE	<10	<10	30	<10	<10
Isooctane	23	42	60	<10	<10

Sludge spiked at 500 μg/Kg d.w.; acetone, 80ºC;10 min.
[a]DE is diatomaceous earth.
%RSD(n=3)<12%.

In the case of in-cell clean-up, florisil, alumina and silica adsorbents were tested. Prior to packing the sample in the cell, the sample was mixed with the adsorbent and diatomaceous earth to prevent clogging of the sample, as

described in Section 2.3. Results with the different adsorbents showed that florisil was the only adsorbent that provides an enhancement in apparent recovery for all compounds, especially in the case of monensin (from 17% to 32%) and salinomycin (from 23% to 48%). Silica seemed not to affect the response of analytes and alumina strongly decreased their response (from 96% to 22% for maduramicin and from 23% to <10% for salinomycin), probably because the analytes were adsorbed by alumina.

Moreover, several solvents were tested for on-cell clean-up. Dichloromethane, ethyl acetate, hexane, methyl tert-butyl ether and isooctane were pushed and pressurised into the cell at ambient temperature for one minute to eliminate interfering substances, especially fats, waxes and oils. Subsequently, the extraction was performed with acetone under the optimised conditions. Results indicate that the analytes were partially extracted with dichloromethane, ethyl acetate, hexane and methyl tert-butyl ether during the clean-up step, resulting in low apparent recoveries. Less analytes were extracted when isooctane was used compared to the other solvents, with the exception of lasalocid and maduramicin, for which the apparent recoveries were reduced by half.

Therefore, an in-cell clean-up with florisil was selected as the best solution for cleaning the sample during the PLE step because it was the only strategy that provided an enhancement in the area for all analytes. Moreover, the on-cell clean-up was discarded.

3.3.2 SPE clean-up

Nowadays, it is common to include an SPE step after the PLE extraction in the method development for reducing interfering substances. The chemical properties of these compounds (low-polarity and polar/ionisable groups) makes it possible for different kinds of sorbent to be used to clean-up the matrix. This can be done by two ways: retaining analytes in the sorbents or retaining the interfering substances. In this section, the recoveries in SPE and the matrix effect were evaluated for each sorbent.

Several SPE cartridges were tested to achieve a clean-up of the sludge extracts. The cartridges were: Oasis HLB (150 mg) from Waters (Wexford, Ireland) and Discovery DPA-6S (500 mg), Discovery DSC-Diol (500 mg) and Supelclean LC-SAX (200 mg) from Supelco (Bellefonte, USA). A vacuum pump connected to a 16-plate manifold from Teknokroma (Sant Cugat del Vallès, Spain) was used for the SPE procedure.

In the first instance, Oasis HLB and Discovery DPA-6S were tested by adapting the method developed in a previous paper [12]. This method is based on the addition of sodium acetate to samples in order to increase the formation of sodium neutral complexes to enhance the retention within the sorbent. Oasis HLB was

chosen based on our previous experience [12] and Discovery DPA-6S sorbent was chosen because it has a resin that can adsorb non-polar and moderately polar compounds containing multiple hydroxyl and carboxyl groups and their use is little reported in the literature.

The spiked organic sludge extracts (500 μg/Kg d.w.) from PLE were evaporated to dryness under nitrogen flow and they were redissolved with 20 mL of aqueous sodium acetate solution (0.5%, w/v; pH 8) before the SPE clean-up. Both cartridges were conditioned with methanol and water prior to sample loading. The clean-up step was performed with water/MeOH (9:1, v/v) and elution was carried out in two different ways for Oasis HLB: with 10 mL of MeOH and 10 mL of MTBE/MeOH (9:1) because the latter mixture does not remove humic acids from the sorbent (supplier information). In the case of Discovery DPA-6S, the elution was performed with 10 mL of acetone in line with the supplier's recommendations. After the elution step, the extracts were evaporated to dryness and they were redissolved with 2 mL of water/MeOH/ACN (2:1:1) with 0.1% FA. The results are shown in Table 4.

The best results were obtained with Oasis HLB with MeOH elution. Recoveries ranged between 73% (narasin) and 92% (salinomycin). However, the matrix effect was not reduced enough for monensin (-61%), lasalocid (-76%) and narasin (-93%). The elution with MTBE/MeOH from Oasis HLB and Discovery DPA-6S did not provide good recoveries (from 30% to 50% for Oasis HLB and from 0% to 15% for Discovery DPA-6S). For these reasons, these strategies were discarded.

Discovery DSC-Diol sorbent was also tested. It is a normal-phase sorbent and it was previously used by Schlüsener *et al.* for the determination of antibiotics (including monensin and salinomycin) in soils [14,15] and liquid manure [18]. Two different methods were used based on the methods proposed by Schlüsener *et al.* to retain the analytes within the sorbent in which the analytes can be dissolved in isooctane or water. The spiked sludge extracts were treated as described above but they were redissolved in 20 mL of isooctane or water prior to SPE. In both cases, the cartridge elution was performed with 10 mL of MeOH. The extract was then evaporated and redissolved with 2 mL of water/MeOH/ACN (2:1:1) with 0.1% FA.

When the PLE extract was redissolved with isooctane, the compounds were retained by DSC-Diol with similar recoveries to those obtained with Oasis HLB (from 73% to 82%) but the improvement in the matrix effect also was not enough for monensin (-77%), lasalocid (-93%) and narasin (-92%).

In the case of the other methodology tested, the compounds were not retained in this sorbent when the extract was redissolved in water (results not shown). A possible explanation of this fact is because the non-polar matrix components

saturate the sorbent because a previous test with a standard solution prepared in water (without interfering substances) demonstrated that the analytes were retained in the sorbent under the conditions tested.

Moreover, a strong anion exchange sorbent (Supelclean LC-SAX) coupled in tandem with Oasis HLB was tested. The spiked PLE extracts were evaporated and redissolved with 20 mL of aqueous sodium acetate solution (0.5%, w/v; pH 8) to form the neutral pseudo-macrocyclic compounds with sodium. The anionic interfering substances may be retained into SAX sorbent and the analytes can be retained in Oasis HLB sorbent. After the loading step, the elution of Oasis HLB cartridge was performed with 10 mL of MeOH. However, the results showed that the analytes are partially retained in the SAX sorbent (by the equilibrium between the complex form (neutral) and non-complex form (anionic)). Thus, the recoveries obtained were not good (below 36% for all compounds) but the matrix effect was greatly improved for all compounds (between -41% and 31 %).

With the results obtained for the different sorbents tested, it was considered that the improvement in the matrix effect is not enough for any of the SPE clean-up and it was decided to discard this option.

Table 4. Recoveries (R, %) and matrix effect (ME, %) for SPE clean-up.

		Monensin	**Maduramicin**	**Salinomycin**	**Lasalocid**	**Narasin**
HLB[a]	R	79	80	92	77	73
	ME	-61	4	-30	-76	-93
HLB[b]	R	35	32	50	36	30
	ME	-80	-69	-68	-92	-95
DPA-6S	R	-	-	-	15	5
	ME	-77	-94	-72	-95	-94
DSC-Diol	R	80	81	74	73	82
	ME	-77	-14	-7	-93	-92
SAX+HLB	R	24	21	36	17	20
	ME	-31	11	31	-41	-61

PLE extract of sludge spiked at 500 μg/Kg d.w.; acetone, 80ºC;10 min.
[a]Elution with 10 mL of MeOH.
[b]Elution with 10 mL of MTBE/MeOH (9:1).

3.3.3 Influence of chromatographic separation

Another way to minimise the matrix effect is by increasing the chromatographic separation between the analytes and the co-eluting interfering substance but this usually results in longer analysis time. However, it is necessary to explore this possibility when the sample clean-up was not effective. The initial

chromatographic method [12] was changed with respect to two stages of the gradient. Firstly, initial conditions were reduced from 70% of organic phase to 50% in order to increase the separation between the analytes and less-retained interfering substances.
Secondly, the slope of gradient was smoothed to extend the retention of the compounds and increase the separation capabilities of the column between the analytes and the most-retained interfering substances.

With this strategy, a slight improvement for all compounds was observed and narasin doubled its area compared to the initial chromatographic separation due to a reduced matrix effect. This compound has the highest ion suppression and so it was decided to use this separation in order to improve the process because the analysis time only increased by 2.5 min.

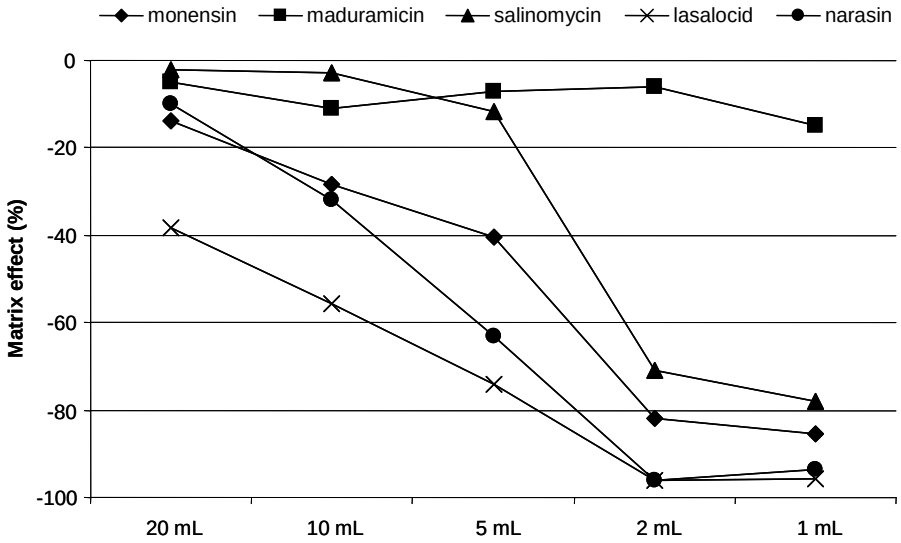

Fig. 2. Matrix effect vs. redissolution volume of PLE extract.

3.3.4 Dilution of PLE extract

Because none of the clean-up strategies studied is fully effective, one solution is the dilution of PLE extracts in order to reduce the matrix effect. In order to ascertain the improvement achieved, different volumes were tested (1, 2, 5, 10 and 20 mL). Florisil was used for the in-cell clean-up in the PLE stage due to the results obtained and the SPE clean-up was avoided in order to save time and the expense involved. The dilutions tested were injected in LC-MS/MS system with the improved chromatographic separation.
However, an increase of dissolution volume of the PLE extracts directly affected the limits of detection

(LODs) and, as a result, it was necessary to choose a compromise value taking the matrix effect into account. Results (Figure 2) show that volumes of 20 and 10 mL gave much less matrix effect than the lower volumes tested, with between -38% (lasalocid) and -2% (salinomycin) and between -56% (lasalocid) and 7% (salinomycin), respectively. Based on these results, 10 mL was chosen as optimal, taking into account the strong reduction of matrix effect. A MRM chromatogram of sludge spiked at 10 µg/Kg (d.w.) is shown in Figure 3.

3.4 Method validation

In order to reduce the differences caused by the matrix effect, an internal standard was initially tested. Based on previous papers [12,17], nigericin was chosen but it had higher ion suppression than the target compounds and, therefore, its use was discarded. To solve the differences caused by the matrix effect it was decided to use a matrix-matched calibration curve as the best approach because isotopic labelled compounds were not commercially available.

Linear range, method limits of quantification (LOQs) and detection (LODs), repeatability (intra-day), reproducibility (day-to-day) and recoveries for the five polyether ionophores involved in this study were determined in order to validate the developed method. All of these parameters are shown in Table 5.

Table 5. Validation parameters for the PLE/LC-MS/MS method.

Compound	LOD µg/Kg (d.w.)	LOQ µg/Kg (d.w.)	Recovery (%, n=6)		Repeatability (intra-day, %RSD, n=6)		Reproducibility (day-to-day, %RSD, n=3)	
			10 µg/Kg (d.w.)	250 µg/Kg (d.w.)	10 µg/Kg (d.w.)	250 µg/Kg (d.w.)	10 µg/Kg (d.w.)	250 µg/Kg (d.w.)
Monensin	0.5	1	88	89	3	2	7	6
Lasalocid	0.5	1	82	80	10	9	12	10
Maduramicin	1	5	94	94	6	4	10	8
Salinomycin	0.5	1	82	90	9	4	12	9
Narasin	0.5	1	98	95	7	4	9	6

Linearity was evaluated by constructing a matrix-matched calibration curve with sludge samples spiked at different concentrations (10 calibration points) from 0.5 to 500 μg/Kg (d.w.) prior to PLE extraction. This calibration curve was used for the quantification of samples and r^2 was higher than 0.999 for all compounds in the range tested. In addition, a non-spiked sludge sample was analysed to subtract the signal of analytes present in sample (only monensin was found at a level near the LOD). LOQs were defined as the lowest point of the calibration curve and LODs corresponded to a signal/noise ratio equal to 3.

Thus, the method was linear from LOQ (1 μg/Kg (d.w.) for all compounds, except for maduramicin which was 5 μg/Kg (d.w.)) to 500 μg/Kg (d.w.). LODs were 0.5 μg/Kg (d.w.) and they were equal for all compounds, except for maduramicin which was 1 μg/Kg (d.w.).

Repeatability (intra-day, n=6) and reproducibility (day-to-day, n=3), expressed as %Relative Standard Deviation (%RSD), were calculated from sludge samples spiked at two levels of concentrations (10 and 250 μg/Kg (d.w.)). Repeatabilities were always below 10% and reproducibilities were better than 12% for all compounds at both concentration levels tested. Recoveries were determined by extracting and analysing a set of 6 sludge samples spiked at the two levels mentioned above (10 and 250 μg/Kg (d.w.)). The results of these spiked samples were compared with two equal sludge samples spiked, at both levels, after the extraction procedure in order to calculate the recoveries for each compound. Recoveries were very similar at the two levels and were between 82% (lasalocid and salinomycin) and 98% (narasin) at the lowest level and between 80% (lasalocid) and 95% (narasin) at the highest level studied.

To the best of our knowledge, this is the first paper that focuses on the determination of polyether ionophores in sludge and the description of a reliable methodology based on PLE/LC-MS/MS for this purpose. Previously, Zhou *et al.* [13] presented a method for the determination of human and veterinary antibiotics in various environmental matrices, including three polyether ionophores (salinomycin, narasin and monensin). They analysed sludge samples but could not apply the multiresidue method to determine polyether ionophores because the recoveries were lower than 10%. Therefore, no data about polyether ionophores in sludge was reported in their study.

4 Method application

The method developed was applied to determine the selected polyether ionophores in twenty-three sludge samples from five different STPs divided as follows: Seven samples from the Tarragona STP, seven from the Reus STP, four from the Blanes STP, three from the Palamós STP and two from the Castell-Platja d'Aro STP.

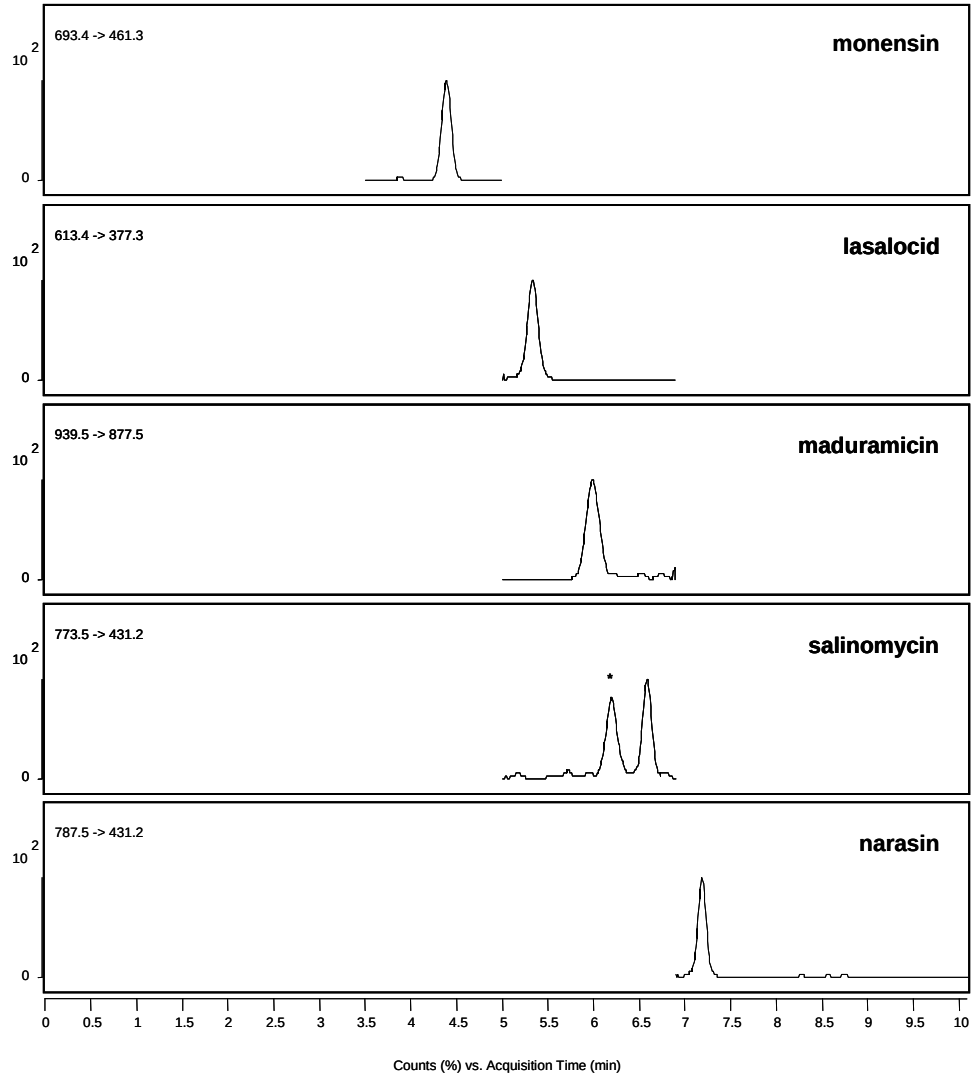

Fig. 3. MRM chromatogram of a spiked sludge at 10 μg/Kg (d.w.).

Two of these compounds, monensin and narasin, were determined in samples from the Tarragona and Reus STPs but they were not detected in samples from the Blanes, Palamós and Castell-Platja d'Aro STPs. The other compounds were not detected in any of the analysed samples. Table 6 shows the results obtained for the positive samples and the two ion ratios for each compound used to confirm the presence of these

compounds under the guidelines of the Commission Decision 2002/657/EC [32]. The maximum accepted tolerances for ion ratio deviations were 20% for the two qualifiers of monensin and 20% and 25% for the two qualifiers for narasin, respectively.

Monensin was detected from <LOQ to 3.5 μg/Kg (d.w.) and narasin from <LOQ to 3.7 μg/Kg (d.w.). The other compounds under study were not detected. The results are in line with previous studies carried out in these STPs, where monensin and narasin were detected in influent and effluent sewage [12] from the Reus STP. In the present study, at least one of the polyether ionophores studied (monensin in six of them) was detected in all of the samples from Reus. In some samples from Tarragona, monensin was determined at levels higher than LOQ and narasin was detected below LOQ in one sample. Hence, the presence of these compounds in the Tarragona STP was lower than in the Reus STP, in accordance with their presence in the sewage at both STPs, as previously reported [12]. For the others STPs studied, residues of these compounds were not found in the Blanes, Castell-Platja d'Aro and Palamós STPs. Nonetheless, studies about polyether ionophores in the sewage at these STPs had not been carried out. Therefore, insufficient information was available about their presence in the water treatment cycle at these STPs. A MRM chromatogram (quantifier and qualifier MRM transitions are included) from sludge sample S4 from the Reus STP is showed in Figure 4.

Table 6. Concentrations of polyether ionophores (μg/Kg (d.w.)) and their respective ion ratios (%) for the sludge samples.

Sample	Monensin		Narasin	
Tarragona STP				
S1	1.7	(94,83)	<LOQ	(82,56)
S2	<LOQ	(80,87)	n.d.	
S3	1.0	(93,85)	n.d.	
Reus STP				
S1	<LOQ	(99,85)	n.d.	
S2	1.0	(94,85)	<LOQ	(63,45)
S3	3.3	(79,88)	2.1	(83,57)
S4	3.5	(95,89)	3.7	(74,34)
S5	2.2	(92,84)	<LOQ	(84,55)
S6	1.8	(88,88)	<LOQ	(74,38)
S7	n.d.		<LOQ	(81,52)

n.d. is not detected.

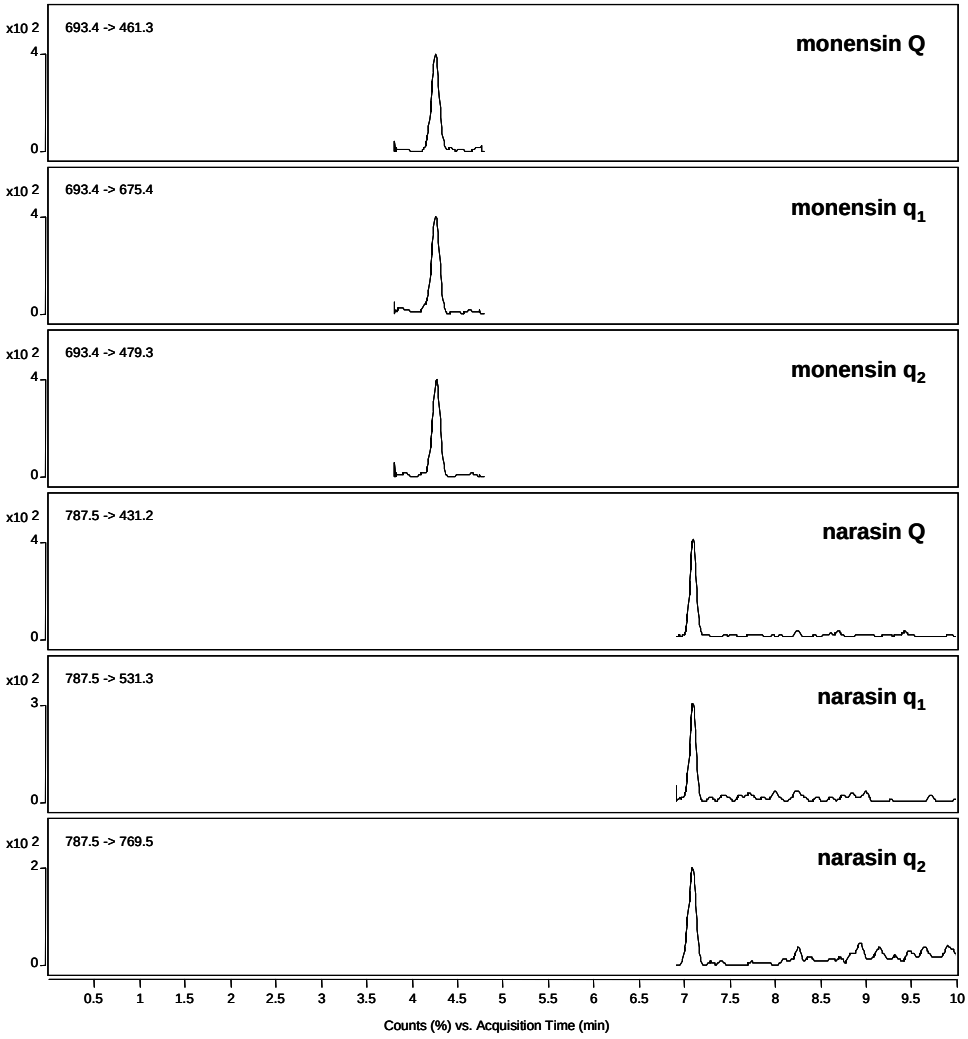

Fig. 4. MRM chromatogram of a sludge sample S4 collected in Reus STP.

The results demonstrate that polyether ionophores are present in sewage sludge and this may result in an environmental risk if this sludge is used as manure. However, an extended discussion about the occurrence of these compounds in sewage sludge has not been developed because the literature about this issue is limited to the paper from Zhou *et al.* [13] as previously mentioned. Therefore, the present paper is the first to describe the occurrence of these compounds in urban sewage sludge and more studies are necessary to identify the

scope of the presence of polyether ionophores in sewage sludge.

5 Conclusions

A reliable and fast analytical method for extracting and analysing five polyether ionophores in sludge samples by PLE/LC-MS/MS was developed. Several clean-up strategies have been tested resulting in the use of florisil in the cell as the best solution combined with an optimised chromatographic method for sludge extracts. The recoveries obtained for the extraction method ranged between 82% and 98% at the low concentration level and between 80% and 95% at the high concentration level. Repeatabilities (intra-day) were from 2% to 10% and reproducibilities (day-to-day) were from 6% to 12% at both concentration levels.
The method provides LODs of 0.5 μg/Kg (d.w.) for all of the compounds, except for maduramicin, which had a LOD of 1 μg/Kg (d.w.). Monensin and narasin were detected in samples from the STPs where these compounds had previously been determined in the sewage.

Acknowledgments

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3.2.3. Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultra-high performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks

UNIVERSITAT ROVIRA I VIRGILI
ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
Pol Herrero Gil
Dipòsit Legal: T 1619-2015

COMPARISON OF TRIPLE QUADRUPOLE MASS SPECTROMETRY AND ORBITRAP-HIGH RESOLUTION MASS SPECTROMETRY IN ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF VETERINARY DRUGS IN SEWAGE: BENEFITS AND DRAWBACKS

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Abstract

This paper presents a comparison of triple quadrupole tandem mass spectrometry (MS/MS) and Orbitrap high resolution mass spectrometry (HRMS) combined to ultra-high performance liquid chromatography (UHPLC) for the determination of glucocorticoids and polyether ionophores in sewage, in order to show the major benefits and drawbacks for each mass spectrometry analyser. Overall, HRMS measurements have enhanced performance in terms of confirmatory capabilities than MS/MS measurements. Moreover, similar limits of quantification (LOQs), limits of detection (LODs), linear range and repeatability for glucocorticoids with both the MS/MS and HRMS methods were compared but in the case of polyether ionophores, slightly better LODs and LOQs were obtained with the HRMS method due to the high sensitivity obtained when diagnostic ions are used for quantification instead of selected reaction monitoring (SRM) transitions for these compounds. The two methods have been applied to the analysis of several influent and effluent sewage samples from sewage treatment plants (STPs) located in the Tarragona region (Catalonia, Spain), showing an excellent correlation between the two methods.

Keywords: *Glucocorticoids; Polyether ionophores; Sewage; UHPLC-(QqQ)MS/MS; UHPLC-(Orbitrap)HRMS.*

1 Introduction

Over the last decade, analytical methods based on triple quadrupole mass spectrometry analysers (QqQ) have undergone significant growth, shown by the large number of applications developed [1-6]. The excellent sensitivity of QqQ analysers when operating in Selected Reaction Monitoring (SRM) mode makes it possible to achieve sub-femtomole detection levels, due to their high signal/noise ratio in comparison to other mass analysers. Although tandem mass spectrometric analysis (MS/MS) may be considered a very selective technique, this selectivity may be overestimated due to the great complexity of some samples, resulting in false positive findings [7-10]. For example, Gallart-Ayala *et al.* [11] discuss the false negative of benzophenone in packaged food samples when QqQ was used. The interference of the Harman compound in terms of the confirmatory SRM transition of benzophenone (it does not meet ion ratio criteria) resulted in a false negative. However, the authors confirmed the positive signal for benzophenone in samples with high resolution mass spectrometry detection.

To adopt a certain criteria to assess the performance of the methods developed, most of the analytical methods implemented follow the guidelines of European Directive 2002/657/EC [12], concerning the performance of analytical methods and the interpretation of results. This Directive is specific to monitor certain substances and residues thereof in live animals and animal products but it is usually adopted in analytical methods to determine emerging organic contaminants in environmental matrices [13]. The Directive requires a minimum of 4 identification points (IP) for the confirmation of substances listed in Group A of Annex I and a minimum of 3 identification points for the substances listed in Group B of Annex I. Moreover, a minimum of at least one ion ratio must be measured. For this reason, a large number of transitions must be monitored simultaneously (in the same time window) in multiresidue analytical methods. This fact compromises the number of points across the peak without losses in sensitivity due to the very low dwell time required, especially when ultra-high performance liquid chromatography (UHPLC) is used, because of the narrow peaks achieved.

Nowadays, the use of high resolution (or ultra-high resolution) mass spectrometers (HRMS) based on Orbitrap mass analysers (100,000-140,000 at 200 m/z in most usual Orbitrap MS instruments and more than 240,000 at 400 m/z in latest generation instruments) is gaining increasing interest in research laboratories, but also routine analysis laboratories for day-to-day analysis [2]. These analysers provide similar detection limits and linear dynamic range to QqQ analysers, in contrast to other medium-high resolution mass spectrometers, such time-of-

flight (TOF) analysers (10,000-40,000 at full width at half maximum (FWHM)) which have certain limitations in terms of determining compounds at (ultra) trace levels [3, 14-16]. HRMS analysers operate in full-scan mode, therefore enabling target, post-target and non-target analysis in a single-run without spending significant amounts of time optimising mass spectrometric conditions for each compound [17]. Moreover, HRMS is suitable for more accurate target analysis, especially for small molecules or compounds that do not have specific SRM transitions, such as loss of water or carbon monoxide [3]. With respect to European Directive 2002/657/EC for HRMS measurements, each ion earns two IPs, so quantification with the molecular ion and the confirmation with one product ion give enough IPs to confirm any substance.

To date, a number of papers have been published with comparisons of MS/MS and HRMS for different kinds of molecules in a range of matrices. For example, De Baere *et al.* [18] compared the QqQ and HRMS methods for the determination of zearalenone in animal plasma, obtaining good correlation between the two methods and better limits of quantification with the HRMS method. Kaufmann *et al.* [10, 19, 20] compared the same kind of MS analysers for the quantification of anthelmintic drug residues in milk and muscle tissue [19], and veterinary drugs in animal tissue and honey [10, 20]. According to their results, HRMS measurements with an Orbitrap mass spectrometer are equally suitable for quantification as QqQ. Vallverdú-Queralt *et al.* [21] evaluated the capabilities of a linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap) and QqQ for the characterisation of tomato polyphenols, with the LTQ-Orbitrap being the technique that offers the greatest benefits for this purpose. The unambiguous assignment of all fragment ions and improved sensitivity and better resolution of mass spectra allowed the identification of certain polyphenols which could not be identified with QqQ. Bruce *et al.* [22] and Henry *et al.* [23] compared the HRMS and MS/MS methods for the determination of vitamin D metabolites in serum [22] and of drugs in patient plasma samples [23], respectively, obtaining excellent correlations and similar LOQs between the two methods. Vanhaecke *et al.* [24] also compared the HRMS and MS/MS methods for the confirmation of anabolic steroids in meat, reporting similar values with both methods in terms of selectivity. However, in this case, the sensitivity of Orbitrap was lower than QqQ. Nevertheless, the comparison of the two aforementioned mass spectrometric techniques for the determination of emerging organic contaminants in environmental waters has scarcely been studied. Gómez-Canela *et al.* [25] recently proposed MS/MS and HRMS as analytical tools for the characterisation of multi-class cytostatic compounds in

environmental waters, but their application is yet to be performed. In addition, Pitarch *et al.* [26] reported the use of gas chromatography (GC) and liquid chromatography (LC), both coupled with a QqQ or with either a TOF or Q-TOF, for the investigation of a wide variety of organic pollutants in sewage. However, this paper focused on their use as complementary analytical techniques rather than a critical comparison between the two. It therefore seems evident that there is a lack of studies in terms of a comparison between MS/MS and HRMS measurements for the determination of emerging organic contaminants in sewage. Furthermore, it is known that sewage contains a wide variety of anthropogenic contaminants and HRMS may be advantageous for its analysis because of the high complexity of the matrix [27].

Therefore, the aim of this paper is to compare the capabilities of HRMS with an Orbitrap analyser with respect to the MS/MS with a QqQ mass analyser for the determination of two types of emerging organic contaminants in sewage. With this in mind, confirmatory capabilities of both mass analysers have been evaluated and both methods have been validated to compare quality parameters. Glucocorticoids and polyether ionophores have been determined in sewage in previous papers [28, 29] using UHPLC-MS/MS and LC-MS/MS methods, and these compounds were selected as candidates for the present study. Synthetic glucocorticoids and polyether ionophores are two examples of veterinary drugs that present an apparent ecotoxicological risk [30-33] and their presence in surface waters [28, 29, 33-44], sewage [28, 29, 33, 34, 44-49], sewage sludge [34, 49-51], soils [40, 52-56] and sediments [38, 39, 44, 52] has been reported.

2 Materials and Methods

2.1 Reagents and standards

Nine standards of glucocorticoids compounds, namely betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide, and five polyether ionophores, namely lasalocid sodium salt solution (100 mg L^{-1} in acetonitrile), maduramicin ammonium salt, monensin sodium salt, narasin and salinomycin, were purchased from Sigma-Aldrich (St. Louis, USA). The chemical structure of glucocorticoids and polyether ionophores are presented in papers [28] and [29], respectively. Fresh stock solutions of individual standards at 1,000 mg L^{-1} (except for lasalocid) were prepared in methanol and stored at -20ºC. A mixed working solution (10 mg L^{-1}) was prepared weekly in methanol and a standard solution with water/acetonitrile (4:1) with formic acid (0.1%) was prepared daily.

Ultrapure water was obtained using an ultrapure water purification system provided by Veolia Water (Sant Cugat del Vallès, Spain). Acetonitrile (ACN)

from Merck (VWR, Llinars del Vallès, Spain) and methanol (MeOH) from Prolabo (VWR, Llinars del Vallès, Spain) were of HPLC grade. Formic acid (FA) for LC-MS analysis was purchased from Merck (Darmstadt, Germany) and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

Sewage samples were collected from the influent and raw effluent of two urban sewage treatment plants (STPs) located in the area of Tarragona. The STPs receive urban sewage and industrial discharges from a population of around 140,000 inhabitants for STP1 and 107,000 for STP2. Both plants use activated sludge biological treatment. The biological oxygen demand (BOD_5) for influent water is about 400 mg L^{-1} at both STPs and the average flow rate is 30,000 m^3 day^{-1} for STP1 and 16,000 m^3 day^{-1} for STP2. All samples were collected by using pre-cleaned amber glass bottles and were filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborough, UK) and stored at 4ºC until analysis, which was always performed within two days after collection. Prior to analysis, the samples were left to reach room temperature.

2.3 Solid-Phase Extraction

The solid-phase extraction method was adapted from previous studies [28, 29]. Sample volumes were 250 mL and 500 mL for influent and effluent sewage samples, respectively. Sodium acetate was added to aqueous samples at a concentration of 500 mg L^{-1} (0.5%, w/v) a few minutes before the extraction. The SPE cartridge used was Oasis HLB (150 mg, 6 cc) from Waters (Wexford, Ireland). A vacuum pump connected to a manifold (Teknokroma, San Cugat del Vallès, Spain) was used for the SPE procedure.

The cartridge was preconditioned with 5 mL of MeOH followed by 5 mL of water, prior to loading the samples into the cartridge at a flow-rate of ~10 mL min^{-1}. Before the elution step, 5 mL of ultrapure water was passed through the sorbent and, afterwards, it was dried under vacuum. The analytes were eluted with 10 mL of MeOH and the eluate was evaporated to dryness under a flow of nitrogen gas. The residue was redissolved in 1 mL of water/acetonitrile (4:1) with 0.1% of formic acid. The extract was filtered with a 0.22 μm PTFE syringe filter before analysis by UHPLC-MS/MS or UHPLC-HRMS.

2.4 UHPLC-(ESI)MS/MS analysis

Chromatographic separation and detection was performed with an Agilent 1200 series chromatograph (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). It was equipped with an automatic injector, a degasser, a binary pump (600 bar) and a column oven. The chromatographic column was a

Zorbax Eclipse XDB-C18 (50 x 4.6 mm, 1.8 μm) (Agilent Technologies). The separation was performed in gradient with ultrapure water/ACN (88:22) 0.1% HCOOH (solvent A) and methanol 0.1% HCOOH (Solvent B) at a flow rate of 1 mL min^{-1}. The gradient started isocratic at 5% of B for 3 min and then increased to 10% in 4 min, at 40% in 3 min, at 85% in 2 min and 95% in 3 min, kept constant for 3 min at 95% and returned to 5% B in 2 min. The fourteen compounds eluted within 15 min. The system was kept at initial conditions for 5 minutes after each run. The oven temperature was kept at 50ºC and the injection volume was 20 μL.

Glucocorticoids were determined under negative electrospray ionisation with $[M+HCOO]^-$ as precursor ions, and polyether ionophores under positive ionisation with $[M+Na]^+$ as precursor ions. The optimal conditions for each compound in the ESI source were adapted from previous studies [28, 29]. The average source conditions were as follows: nebuliser pressure of 40 psi, drying gas (N_2) flow rate of 12 L min^{-1}, drying gas temperature of 350ºC, and capillary voltage of -2 kV and +3.5 kV for negative and positive ionisation, respectively. Cone voltage and collision energies were optimized for each compound to obtain three SRM transitions following the guidelines of European Directive 2002/657/EC [12] to obtain 5.5 IPs. The most intense SRM transition was used for quantification and the other two were used for the confirmation of analytes (Table 1).The ratios between the two transitions were used for confirmation purposes and are also shown in Table 1. Three time windows were used: 2-7 min (prednisone, prednisolone, hydrocortisone and cortisone), 7-12 min (methylprednisolone, betamethasone, flumethasone, dexamethasone and triamcinolone acetonide) and 12-20 min (monensin, lasalocid, maduramicin, salinomycin and narasin).

2.5 UHPLC-(ESI)HRMS analysis

An Accela 1250 UHPLC chromatograph coupled to a single-stage Orbitrap/Exactive mass analyser equipped with a high-energy collisional dissociation cell (HCD) from Thermo Fisher Scientific (Bremen, Germany) was used for UHPLC-HRMS measurements. It was equipped with a quaternary pump (1,250 bar) and an Accela Autosampler, consisting of an automatic injector (refrigerated to 5ºC) and a column oven (heated to 50ºC). The electrospray interface was a heated electrospray ionisation source (HESI-II). The chromatographic column and conditions were the same as in the UHPLC-(ESI)MS/MS method (Section 2.4).

In order to optimise HRMS measurements, a mixture of all of the compounds were infused into the source with a syringe pump connected to the column flow conditions. The diagnostic ions $[M+HCOO]^-$ for glucocorticoids and

Table 1. UHPLC-MS/MS acquisition parameters in SRM mode. C.V. is cone voltage and C.E. is collision energy. Quantification SRM transition is in bold.

Compounds	**C.V. (V)**	**Precursor ion (m/z)**	**C.E. (eV)**	**Product ions (m/z)**	**Ion ratio (%)**
Glucocorticoids					
Prednisone	100	**403.2** $[M+HCOO]^-$	10	**327.2** $[M\text{-}H\text{-}CH_2O]^-$	
			15	299.1 $[M\text{-}H\text{-}CH_2O\text{-}CO]^-$	17
			30	285.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_2CO]^-$	10
Prednisolone	100	**405.2** $[M+HCOO]^-$	10	**329.2** $[M\text{-}H\text{-}CH_2O]^-$	
			30	295.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$	16
			30	280.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}\cdot CH_3]^-$	13
Cortisone	100	**405.2** $[M+HCOO]^-$	10	**329.2** $[M\text{-}H\text{-}CH_2O]^-$	
			35	297.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$	20
			35	282.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}\cdot CH_3]^-$	9
Hydrocortisone	100	**407.2** $[M+HCOO]^-$	10	**331.2** $[M\text{-}H\text{-}CH_2O]^-$	
			15	301.2 $[M\text{-}H\text{-}CH_2O\text{-}CO]^-$	20
			30	311.2 $[M\text{-}H\text{-}CH_2O\text{-}H_2O]^-$	19
Methylprednisolone	110	**419.2** $[M+HCOO]^-$	10	**343.2** $[M\text{-}H\text{-}CH_2O]^-$	
			35	309.2 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O]^-$	23
			35	294.1 $[M\text{-}H\text{-}CH_2O\text{-}CH_4\text{-}H_2O\text{-}\cdot CH_3]^-$	19

Table 1. (Cont.).

Compounds	C.V. (V)	Precursor ion (m/z)	C.E. (eV)	Product ions (m/z)	Ion ratio (%)
Flumethasone	110	**455.2** $[M+HCOO]^-$	15	**379.2** $[M-H-CH_2O]^-$	
			35	305.2 $[M-H-CH_2O-CH_4-2HF-H_2O]^-$	22
			30	325.2 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	12
Betamethasone	110	**437.2** $[M+HCOO]^-$	15	**361.2** $[M-H-CH_2O]^-$	
			35	307.2 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	27
			35	292.1 $[M-H-CH_2O-CH_4-HF-H_2O-{}^{\cdot}CH_3]^-$	19
Dexamethasone	110	**437.2** $[M+HCOO]^-$	15	**361.2** $[M-H-CH_2O]^-$	
			35	307.2 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	22
			35	292.1 $[M-H-CH_2O-CH_4-HF-H_2O-{}^{\cdot}CH_3]^-$	15
Triamcinolone	110	**479.2** $[M+HCOO]^-$	15	**413.2** $[M-H-HF]^-$	
			20	337.2 $[M-H-C_2H_6CO-HF-H_2O]^-$	99
			10	375.2 $[M-H-C_2H_6CO]^-$	77
Polyether ionophores					
Monensin	180	**693.4** $[M+Na]^+$	55	**461.3** $[M+Na-2H_2O-C_{11}H_{16}O_3]^+$	
			55	479.3 $[M+Na-H_2O-C_{11}H_{16}O_3]^+$	91
			35	675.4 $[M+Na-H_2O]^+$	69
Lasalocid	180	**613.4** $[M+Na]^+$	35	**377.3** $[M+Na-C_{13}H_{16}O_4]^+$	
			30	577.3 $[M+Na-2H_2O]^+$	75
			20	595.4 $[M+Na-H_2O]^+$	8

Table 1. (Cont.).

Compounds	C.V. (V)	Precursor ion (m/z)	C.E. (eV)	Product ions (m/z)	Ion ratio (%)
Maduramicin	180	**939.5** $[M+Na]^+$	35	**877.5** $[M+Na\text{-}H_2O\text{-}CO_2]^+$	
			50	895.5 $[M+Na\text{-}CO_2]^+$	14
			70	859.5 $[M+Na\text{-}2H_2O\text{-}CO_2]^+$	13
Salinomycin	180	**773.5** $[M+Na]^+$	55	**431.2** $[M+Na\text{-}C_{19}H_{34}O_5]^+$	
			45	531.3 $[M+Na\text{-}C_{13}H_{22}O_4]^+$	36
			35	755.5 $[M+Na\text{-}H_2O]^+$	8
Narasin	180	**787.5** $[M+Na]^+$	50	**431.2** $[M+Na\text{-}C_{20}H_{36}O_5]^+$	
			50	531.3 $[M+Na\text{-}C_{14}H_{24}O_4]^+$	40
			40	769.5 $[M+Na\text{-}H_2O]^+$	9

$[M+Na]^+$ for polyether ionophores were monitored to obtain the best average parameters that affect the performance of ionisation in each mode, positively or negatively, in full scan at a resolution of 50,000 FWHM over a mass-range of 100-1,000 Da. Thus, the optimised value for spray voltage was 3 kV and 3.5 kV, for capillary voltage was -40 V and 40 V, for tube lens voltage was -110 V and 160 V, and for skimmer voltage was -18 V and 18 V, in negative and positive mode, respectively. The sheath gas was fixed at 75 AU and the auxiliary gas at 20 AU. The transfer tube and heater temperature were set at 350ºC in both ionisation modes.

Two time windows were used, one in negative mode (0-11.5 min) and the other in positive mode (11.5-20 min), with two scan events in each time window. The scan events were as follows: one in full scan mode (at 50,000 FWHM (2Hz) with 250 ms of injection time) and the other with all ion fragmentation mode (at 10,000 FWHM (10 Hz) with 50 ms of injection time) at 30 eV and 60 eV in the HCD cell in negative and positive ionisation mode, respectively. The diagnostic ions (with an mass extraction window of 5 ppm) were used for quantification and three fragment ions (and their corresponding ion ratios (Table 2)) were also measured to obtain 8 IPs, which complies with the guidelines of European Directive 2002/657/EC [12], and were used for confirmation purposes.

Table 2. UHPLC-HRMS acquisition parameters in full-scan mode. Fragment ions are obtained at 30 eV for glucocorticoids and 60 eV for polyether ionophores.

Compounds	**Diagnostic ion (m/z)**	**Fragment ions (m/z)**	**Ion ratio (%)**
Glucocorticoids			
Prednisone	403.17623 $[M+HCOO]^-$	327.16018 $[M-H-CH_2O]^-$	28
		285.14962 $[M-H-CH_2O-CH_2CO]^-$	10
		299.14962 $[M-H-CH_2O-CO]^-$	5
Prednisolone	405.19188 $[M+HCOO]^-$	329.17583 $[M-H-CH_2O]^-$	14
		295.13397 $[M-H-CH_2O-CH_4-H_2O]^-$	9
		280.11049 $[M-H-CH_2O-CH_4-H_2O-{\cdot}CH_3]^-$	6
Cortisone	405.19188 $[M+HCOO]^-$	329.17583 $[M-H-CH_2O]^-$	41
		301.18092 $[M-H-2CO]^-$	7
		311.16527 $[M-H-CO-H_2O]^-$	6
Hydrocortisone	407.20753 $[M+HCOO]^-$	329.17583 $[M-H-H_2-CH_2O]^-$	14
		331.19148 $[M-H-CH_2O]^-$	12
		297.14962 $[M-H-H_2-CH_2O-CH_4-H_2O]^-$	10
Methylprednisolone	419.20753 $[M+HCOO]^-$	343.19148 $[M-H-CH_2O]^-$	13
		309.14962 $[M-H-CH_2O-CH_4-H_2O]^-$	10
		294.12614 $[M-H-CH_2O-CH_4-H_2O-{\cdot}CH_3]^-$	7

Table 2. (Cont.).

Compounds	Diagnostic ion (m/z)	Fragment ions (m/z)	Ion ratio (%)
Flumethasone	455.18868 $[M+HCOO]^-$	379.17264 $[M-H-CH_2O]^-$	27
		305.11832 $[M-H-CH_2O-CH_4-2HF-H_2O]^-$	11
		325.12455 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	7
Betamethasone	437.19811 $[M+HCOO]^-$	361.18206 $[M-H-CH_2O]^-$	23
		307.13397 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	16
		292.11049 $[M-H-CH_2O-CH_4-HF-H_2O-{}^{\cdot}CH_3]^-$	7
Dexamethasone	437.19811 $[M+HCOO]^-$	361.18206 $[M-H-CH_2O]^-$	25
		307.13397 $[M-H-CH_2O-CH_4-HF-H_2O]^-$	15
		292.11049 $[M-H-CH_2O-CH_4-HF-H_2O-{}^{\cdot}CH_3]^-$	8
Triamcinolone	479.20867 $[M+HCOO]^-$	337.14453 $[M-H-C_2H_6CO-HF-H_2O]^-$	7
		413.19696 $[M-H-HF]^-$	5
		375.16133 $[M-H-C_2H_6CO]^-$	5
Polyether ionophores			
Monensin	693.41839 $[M+Na]^+$	675.40672 $[M+Na-H_2O]^+$	16
		461.28680 $[M+Na-2H_2O-C_{11}H_{16}O_3]^+$	13
		479.32973 $[M+Na-H_2O-C_{11}H_{16}O_3]^+$	11
Lasalocid	613.37109 $[M+Na]^+$	377.26595 $[M+Na-C_{13}H_{16}O_4]^+$	22
		595.36064 $[M+Na-H_2O]^+$	10
		559.33915 $[M+Na-3H_2O]^+$	8

Table 2. (Cont.).

Compounds	Diagnostic ion (m/z)	Fragment ions (m/z)	Ion ratio (%)
Maduramicin	939.52877 $[M+Na]^+$	877.52643 $[M+Na-H_2O-CO_2]^+$	88
		859.51648 $[M+Na-2H_2O-CO_2]^+$	66
		895.53970 $[M+Na-CO_2]^+$	20
Salinomycin	773.48103 $[M+Na]^+$	431.23960 $[M+Na-C_{19}H_{34}O_5]^+$	24
		531.32827 $[M+Na-C_{13}H_{22}O_4]^+$	18
		755.46844 $[M+Na-H_2O]^+$	6
Narasin	787.49668 $[M+Na]^+$	431.23958 $[M+Na-C_{20}H_{36}O_5]^+$	27
		531.32827 $[M+Na-C_{14}H_{24}O_4]^+$	19
		769.48444 $[M+Na-H_2O]^+$	7

3 Results and discussion

3.1 Ultra-high performance liquid chromatography

The UHPLC separation was optimised from previous studies [28, 29] in which glucocorticoids and polyether ionophores were determined, separately, in sewage. The first part of gradient comprises the separation of glucocorticoids the second part the separation of polyether ionophores, which are subject to higher retention than glucocorticoids. The addition of acetonitrile in the aqueous mobile phase is due to the improvement in the separation of betamethasone and dexamethasone epimers, which cannot be differentiated by mass spectrometry.

3.2 Mass spectrometry

For the QqQ analyser, the parameters that affect the performance of the ESI interface and collision energies were individually optimised for each compound in previous papers [28, 29], and are described in Section 2.4.
For the Orbitrap analyser, the optimisation of ionisation performance in HESI source (HESI-II) was performed by the infusion of a mixed solution of all of the compounds under chromatographic parameters (flow and mobile phase composition). The probe position adjustment (side-to-side (-1 to +1), vertical (C or D) and micrometer (1 to 2)), capillary and transfer tube temperature (250 to 450ºC) and

sheath (50 to 100 AU) and auxiliary gas (0 to 50 AU) were chosen as a compromise between the maximum formation efficiency of diagnostic ions for all compounds. Spray voltage (2 to 5 kV), capillary voltage (±10 to ±100 V), tube lens voltage (±50 to ±200 V) and skimmer voltage (±5 to ±50 V) were selected for each ionisation mode as a compromise between the maximum formation efficiency of diagnostic ions of each compound family. The values that provided the best response are described in Section 2.5. Collision energies (5 to 70 eV) in HCD were optimised to observe at least two known fragment ions for each compound in all ion fragmentation spectra for confirmatory purposes.

3.3 Mass spectral characterisation

3.3.1 Glucocorticoids

The mass spectral characterisation of glucocorticoids with QqQ was extensively reported in a previous paper [28] and it was similar to that obtained with the Orbitrap analyser. To summarise, glucocorticoids form different precursor ions depending on the mobile phase and ionisation mode used [28, 45, 57]. Thus, in the positive ionisation mode, $[M+H]^+$ and $[M+Na]^+$ were the most intense ions, but $[M+H-H_2O]^+$ or $[M+H-HF]^+$ were also observed. In the negative ionisation mode, glucocorticoids showed formate adduct $[M+HCOO]^-$ as the most abundant precursor ion when formic acid was added to the mobile phase [58], although chloride adducts $[M+Cl]^-$ also appeared but at low levels of abundance. In addition, these compounds present in-source fragmentation, more so in positive mode, and their spectral profiles were similar for ESI (QqQ) and HESI (Orbitrap) sources.

Subsequently, the fragmentation of these precursor ions was studied. At low collision energies the precursor $[M+H]^+$ produced many fragments with low intensity and no fragmentation of the sodium adduct $[M+Na]^+$ was observed. Therefore, the positive ionisation mode was not suitable for these compounds because of the formation of multiple precursors and multiple low intense product ions. The fragmentation of formate adduct precursors $[M+HCOO]^-$ generated $[M-H-CH_2O]^-$ as the most abundant product ion, and other less intense fragments linked to losses of water due to the high number of hydroxyl groups present in these compounds or hydrogen fluoride for molecules which have fluorine atoms in their structure (Tables 1 and 2). Moreover, losses of methyl radical groups were also observed. A full-scan and all ion fragmentation spectra acquired with HRMS (HCD cell) for betamethasone are shown in Figure 1. Triamcinolone acetonide had different fragmentation pathways compared to the other glucocorticoids because of its different structure in C16 to C19 carbons. Therefore, negative ionisation was selected for glucocorticoids which is in line with Antignac *et al.* [59], who indicated that negative mode is more specific for

corticosteroid ionisation because the loss of formaldehyde is very characteristic for these compounds and enhanced sensitivity is achieved because all of the signal corresponding to the target compound is concentrated on a single m/z value $[M+HCOO]^-$.

3.3.2 Polyether ionophores

As occurred before, MS spectra features for polyether ionophores obtained with QqQ were similar to those obtained with HRMS. Since the capacity to complex alkali metals, especially sodium, polyether ionophores were ionised in positive mode as sodium adducts $[M+Na]^+$. Ammonium adduct $[M+NH_4]^+$ and protonated molecule with a loss of water $[M+H-H_2O]^+$ were also formed but without a significant relevance in mass spectra. The fragmentation pathways of polyether ionophores followed two routes. Losses of water or carbon dioxide were common, but fragmentations involving higher mass losses were not very clear. However, some authors [43] have suggested that these reactions are caused by a gas-phase *β*-cleavage reaction for polyether ionophores that contain a ketone group in their structure. A full-scan and all ion fragmentation spectrums acquired with HRMS (HCD cell) for monensin is shown in Figure 2.The mass fragments obtained (Table 2) were more intense in HRMS (HCD cell) than those obtained with MS/MS (QqQ), with detection and quantification limits being improved with HRMS.

The negative ionisation mode was only slightly effective for these compounds because of their affinity to complex alkali metals when polyether ionophores are deprotonated. Under these conditions, equilibrium was achieved between complex form (neutral and non-detectable in MS) and non-complex form (anionic and detectable in MS as $[M-H]^-$), reducing the response of these precursor ions in MS.

3.4 Solid-phase extraction

The solid-phase extraction method for both groups of compounds was optimised from the optimal conditions for polyether ionophores reported in a previous study [29] in which an increase in the recovery was obtained with Oasis HLB when 0.5% (w/v) of sodium acetate was added to samples. The addition of sodium acetate to sewage samples gives a pH ~8. At this pH, polyether ionophores are deprotonated which increases their ability to complex sodium resulting in the formation of neutral complexes which are more retained in Oasis HLB sorbent. Thus, the SPE method (Section 2.3) was applied to extract both groups of compounds in one single step because the recovery of glucocorticoids was not affected by changes in the sample pH [28]. Therefore, 500 mL of effluent sewage spiked at 20 ng L^{-1} was extracted with the aforementioned method and analysed by the UHPLC-MS/MS method to calculate the recovery yield. The recoveries obtained for

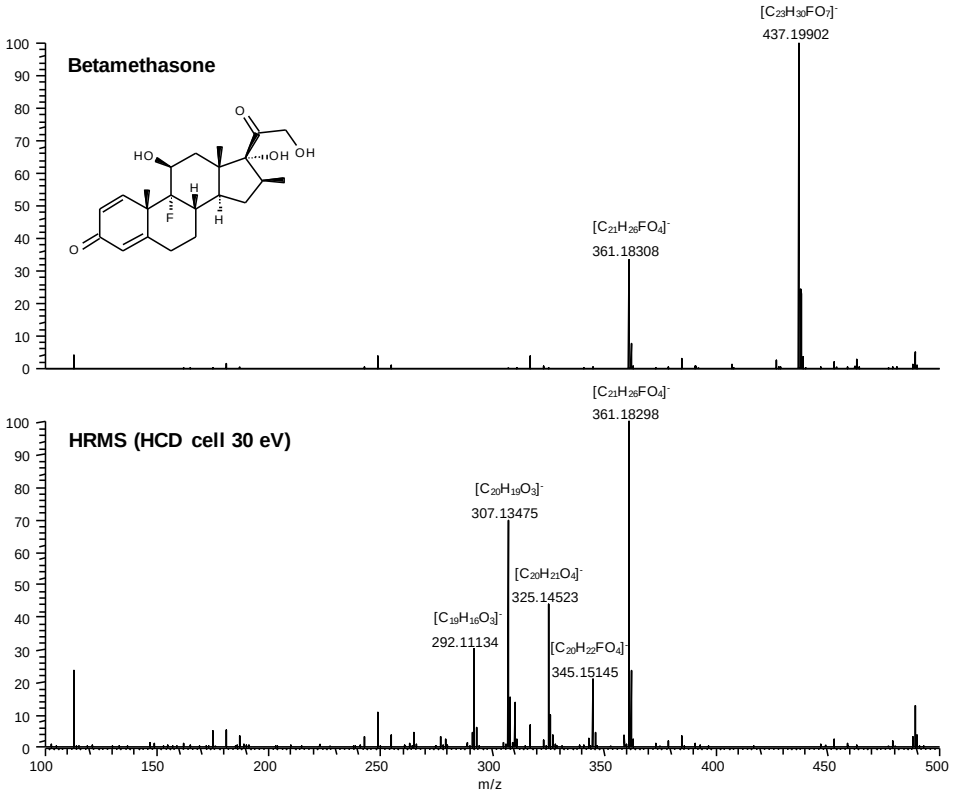

Fig. 1. Full-scan HRMS spectrum and all ion fragmentation spectrum (HCD cell at 30 eV) of betamethasone with Orbitrap analyser (mass error of diagnostic ion: 2.071 ppm).

glucocorticoids and polyether ionophores were similar to those obtained in a previous studies [28, 29] and were from 87% for prednisolone to 99% for hydrocortisone and from 88% for lasalocid to 95% for maduramicin. These results demonstrate the applicability of this SPE method for the simultaneous analysis of glucocorticoids and polyether ionophores.

Moreover, influent (400 ng L^{-1}) and effluent (200 ng L^{-1}) sewage samples spiked after the SPE procedure were analysed by the UHPLC-MS/MS and UHPLC-HRMS methods in order to evaluate the matrix effect with each method. Since the matrix effect in MS occurs during ionisation, differences in the matrix effect can be expected with both methods because the interfaces of both instruments differ from each other (orthogonal ESI in QqQ and heated-electrospray (HESI) at 45ºC in Orbitrap). However, it was found that both MS methods showed very similar matrix effects, with differences lower than 15% between

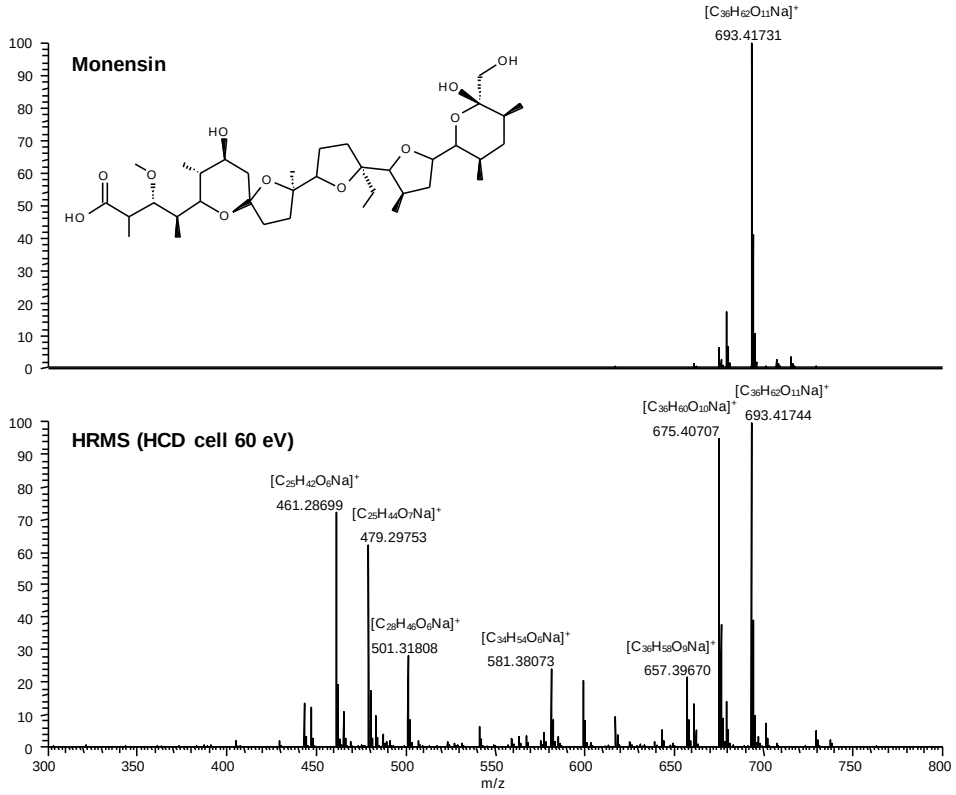

Fig. 2. Full-scan HRMS spectrum and all ion fragmentation spectrum (HCD cell at 60 eV) of monensin with Orbitrap analyser (mass error of diagnostic ion: -1.549 ppm).

the two. Thus, glucocorticoids underwent ion suppression of approximately -20% and polyether ionophores underwent ion enhancement of up to 50%. The results obtained were similar to those reported previously for glucocorticoids [28] and for polyether ionophores [29] with MS/MS.

3.5 Method validation

The UHPLC-MS/MS and UHPLC-HRMS methods were validated in order to ascertain the benefits of each method developed. A matrix matched calibration curve was used both for influent and effluent sewage because isotopic labelled compounds were not commercially available for polyether ionophores.

Linear range, limits of quantification (LOQ) and detection (LOD), and repeatability (expressed as relative standard deviation (%RSD, n=3)) were calculated for the compounds under study with both methods. In all cases, a blank sample was analysed to

evaluate the presence of these compounds in order to subtract their response in calibration points, whenever necessary. The linear range was evaluated by spiking influent and effluent samples between 1 and 1,000 ng L^{-1} and between 0.5 and 500 ng L^{-1}, respectively. The methods were linear between LOQ to the highest concentration level tested for all compounds in both matrices. LOQs were assigned at the lowest point of the calibration curve and LODs were evaluated by spiking blank sewage samples until a signal/noise ratio higher than 3 times and signal intensity higher than $1x10^3$ in Orbitrap analyser which accomplish in both cases the ion ratio criteria showed in Tables 1 and 2, respectively. The accepted tolerances are those reported in the Directive 2002/657/EC [45]. For cortisone and hydrocortisone which were present in all influent samples, their LODs were estimated from their MS response.

For example, LODs and LOQs obtained for UHPLC-MS/MS and UHPLC-HRMS methods with influent sewage samples are shown in Table 3. The results obtained with both methods were very similar for glucocorticoids, with LODs around 5 ng L^{-1} and LOQs around 20 ng L^{-1}, except in the case of triamcinolone acetonide, which showed better LODs and LOQs with HRMS measurements because of their different fragmentation pathways compared to the other glucocorticoid compounds. For polyether ionophores, the LODs and LOQs obtained were slightly better with the HRMS method. This fact is because the parent ion is used for the quantification instead of an SRM transition. For effluent sewage samples, the values obtained were slightly lower than for influent samples due to the higher sample volume used. The LODs and LOQs ranged from 1 to 10 ng L^{-1} and from 2.5 to 20 ng L^{-1}, respectively, depending on the compound and method employed. Nonetheless, the LODs and LOQs values are highly dependent on the instrument used and therefore, these values cannot be used as the main parameter to compare the benefits provided by each mass analyser. In the present study, the QqQ analyser is currently in use in many laboratories for routine analysis and it is interesting to compare the LODs and LOQs provided by one of the most established instrumentation (Agilent 6410) with the capabilities offered by one of the emerging mass analyser in routine analysis laboratories (Orbitrap/Exactive).

The repeatability of the methods was evaluated by analysing three replicates of an influent sample (%RSD, n=3) with both methodologies, the results of which are also shown in Table 3. Repeatability values achieved were less than 9% for the compounds determined in the sample (prednisone, prednisolone, cortisone, hydrocortisone, methylprednisolone, and betamethasone) with both methods. Dexamethasone and triamcinolone acetonide were detected below of their LOQ and the other compounds were not detected.

Moreover, to determine the repeatability (Table 3) for the compounds not present in the sample used above, three replicates of an influent spiked sample (%RSD, 20 ng/L) were analysed by SPE/UHPLC-HRMS. As shown in Table 3, the repeatability values for all compounds were less than 8%. Based on the results obtained in this paper, HRMS measurements with Orbitrap type analysers showed similar sensitivity, linear range and repeatability to QqQ when highly complicated matrices are analysed.

Table 3. Limits of detection (LOD) and quantification (LOQ) and repeatability for MS/MS (QqQ) and HRMS (Orbitrap) methods calculated from influent sewage samples.

Compounds	LOD (ng L^{-1})		LOQ (ng L^{-1})		Repeatability (%RSD, n=3)		
	QqQ	Orbitrap	QqQ	Orbitrap	QqQ	Orbitrap	Orbitrap*
Glucocorticoids							
Prednisone	5	5	20	20	1	3	4
Prednisolone	5	5	20	20	3	7	3
Cortisone	5	5	20	20	4	5	6
Hydrocortisone	5	5	20	20	3	6	3
Methylprednisolone	5	5	20	20	7	9	5
Flumethasone	5	5	20	20	n.d	n.d	7
Betamethasone	5	5	20	20	6	7	8
Dexamethasone	5	5	20	20	<LOQ	<LOQ	2
Triamcinolone	10	5	50	10	n.d.	<LOQ	6
Polyether ionophores							
Monensin	2.5	1	10	2.5	n.d.	n.d.	1
Lasalocid	2.5	2.5	10	5	n.d.	n.d.	3
Maduramicin	10	5	50	10	n.d.	n.d.	5
Salinomycin	2.5	2.5	10	5	n.d.	n.d.	5
Narasin	2.5	1	10	2.5	n.d.	n.d.	2

*Spiked at 20 ng/L

4 Application to environmental samples

Different samples were analysed by both methods to determine glucocorticoids and polyether ionophores in eight influent and eight effluent sewage samples from two STPs located in the Tarragona region. The average concentration determined for each compound with HRMS method is summarised in Table 4, including the maximum and minimum values found and the average value of mass error measured which, in all cases, was lower than 5 ppm. The ion ratios between monitored SRM transitions for MS/MS method (Table 1) and between fragment ions and diagnostic

Table 4. Concentration found (ng L^{-1}) of glucocorticoids and polyether ionophores investigated in sewage samples and the mass error measured for diagnostic ions by UHPLC-Orbitrap-HRMS method.

Compounds	Mass error (ppm)	STP1						STP2					
		Influent			Effluent			Influent			Effluent		
		Avg. (ng L^{-1})	Max.	Min.	Avg. (ng L^{-1})	Max.	Min.	Avg. (ng L^{-1})	Max.	Min.	Avg. (ng L^{-1})	Max.	Min.
Glucocorticoids													
Prednisone	3.83	14.9	21.2	<20	n.d.	-	-	17.4	24.2	<20	n.d.	-	-
Prednisolone	3.29	25.7	34.4	20.8	<10	<10	<10	33.7	52.5	21.0	<10	<10	<10
Cortisone	3.51	196.8	224.3	178.8	n.d.	-	-	207.0	281.3	89.2	n.d.	-	-
Hydrocortisone	4.08	275.7	316.0	222.8	<10	<10	<10	244.0	273.5	208.3	<10	<10	<10
Methylprednisolone	4.18	<20	<20	<20	<10	<10	<10	32.1	82.7	<20	<10	<10	<10
Betamethasone	2.80	22.2	28.4	<20	<10	<10	<10	43.4	121.9	<20	9.4	12.6	<10
Dexamethasone	3.44	<20	<20	<20	n.d.	-	-	<20	<20	<20	n.d.	-	-
Triamcinolone	4.38	<10	<10	<10	n.d.	-	-	<10	<10	<10	n.d.	-	-
Polyether ionophores													
Monensin	0.80	n.d.	-	-	n.d.	-	-	35.6	39.6	31.6	11.5	27.0	1.8

*Mass error is the average value from compounds detected in analysed sewage samples.

ions for HRMS method (Table 2) were used for the confirmation of the compounds as described in Sections 2.4 and 2.5. The concentrations found agree with previous studies carried out in the same STPs [28, 29]. All of the glucocorticoids, except flumethasone, were detected in influent sewage samples and some of them were determined at concentrations higher than LOQ (prednisone, prednisolone, cortisone, hydrocortisone and betamethasone) in both plants. Methylprednisolone was determined in one influent sewage sample from STP2. Monensin was the only polyether ionophore determined in a single influent sample from STP2.

In the case of effluent sewage samples, the presence of these compounds was low. Normally, some glucocorticoids were found at levels below of LOQ (prednisolone, hydrocortisone, methylprednisolone and betamethasone) and monensin was determined in all of the effluent sewage samples from STP2. The occurrence of monensin in effluent sewage from this STP, and not in STP1, was previously reported [29] as a result of the agricultural and farming discharge in STP2. The most abundant compounds were cortisone and hydrocortisone, which are endogenous glucocorticoids. The results denote a high elimination efficiency (or transformation) for prednisone and cortisone. This fact may be related to the presence of a ketone group in C11, in contrast to the hydroxyl group, which the other glucocorticoids have. In addition, the results obtained for both plants are similar and an extended discussion of the presence of glucocorticoids and polyether ionophores in sewage samples can be found in previous studies [28, 29].

Regarding to the results obtained with UHPLC-MS/MS and UHPLC-HRMS methods, a correlation curve with concentrations measured of the same samples with both methods is presented in Figure 3. The results were comparable between MS/MS and HRMS methods as can be demonstrate by the linearity of the points represented (r^2>0.996) in the aforementioned graph. The slope of regression line shows that no tendency of supra-estimation or sub-estimation is present with MS/MS or HRMS methods. A SRM chromatogram and a HRMS chromatogram of an influent sewage sample are showed in Figure 4. The results of correlation with both methods and the similar quality parameters (LODs, LOQs, linearity and repeatability) showed, demonstrate that HRMS measurements with Orbitrap analysers are a potential tool for emerging water contaminants trace analysis.

In addition, confirmatory benefits provided by HRMS versus MS/MS can be seen in the determination of lasalocid in sewage samples. Signals for quantification and confirmation SRM transitions of lasalocid were observed with UHPLC-MS/MS method and they were not observed with UHPLC-HRMS method. Moreover, second confirmatory SRM

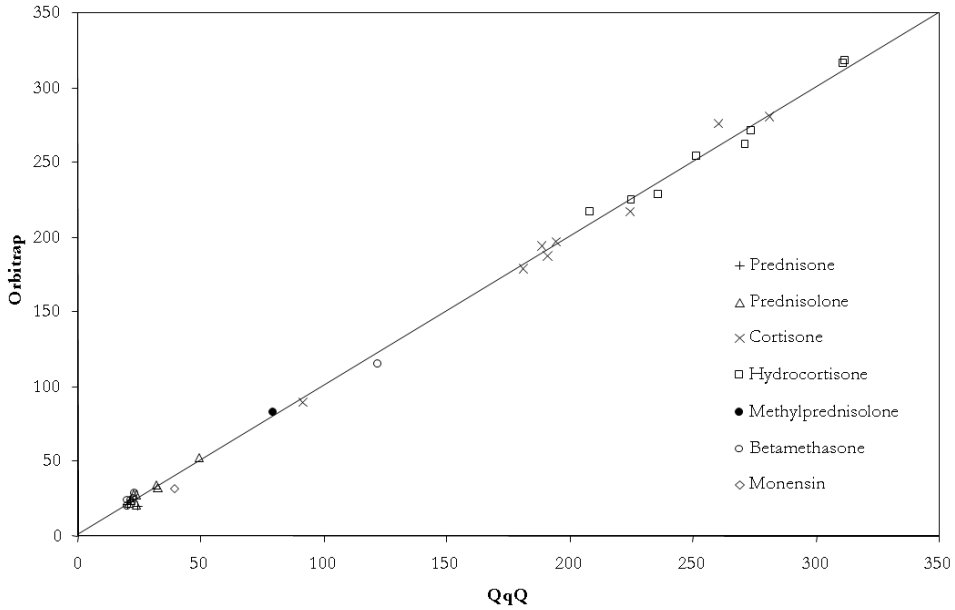

Fig. 3. Correlation graph of concentrations (ng L^{-1}) determined of studied compounds with QqQ and Orbitrap analysers in STPs influent samples.

transition (613.4>577.3) was much higher (100-folds more) than it was expected and not accomplished ion ratio criteria. If HRMS spectra of the sample is carefully examined in the region near to precursor ion of lasalocid ($[M+Na]^+$ with 613.37109 m/z), others mass signals (612.89722 m/z and 613.39862 m/z) were observed nearly of theoretical exact mass of lasalocid. The mass signals observed corresponded to isotopic signals (612.89722 m/z for ^{13}C isotope and 613.39862 m/z for ^{18}O isotope) of a sodium adduct double-charged molecule $[M+H+Na]^{2+}$ (612.39557 m/z). A mass signal corresponding to the same non-sodiate double-charged molecule $[M+2H]^{2+}$ was also present in HRMS spectra (590.38257m/z). This mass was assigned at a molecular formula of $C_{61}H_{110}O_{21}$ (-2.857 ppm). This structure may be a humic substance due to the high concentration of these compounds in sewage. Due to the great number of alcohols and other oxo groups in humic substances is probably that their product ions are formed by loses of water or carbon dioxide and then, interfere the lasalocid SRM transitions. If only one confirmatory SRM transition was monitored (obtaining 4 IP) these could be a false positive for lasalocid with MS/MS method. With HRMS with a mass extraction window of 5 ppm, this compound has not been detected in the samples.

The use of HRMS in full-scan could

prevent false results when environmental samples are analysed because isobaric interferences from the matrix can be distinguished from the target compounds and identified. Therefore, HRMS offers higher confirmatory confidence and selectivity than low resolution MS/MS methods with excellent quantitative capabilities.

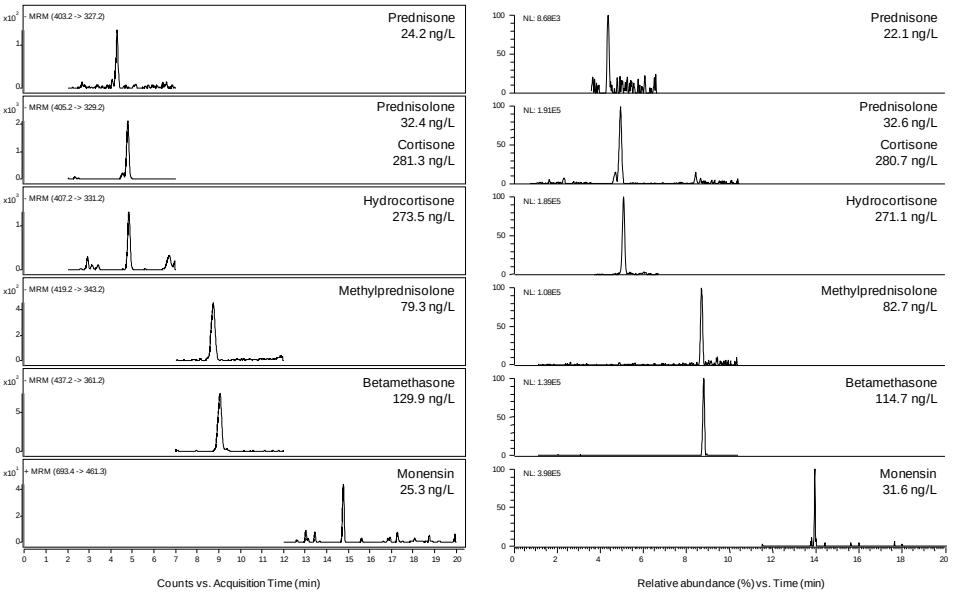

Fig. 4. SRM chromatogram (left) and HRMS chromatogram (right) of an influent sewage sample from STP2.

5 Conclusions

Two analytical methods based on ultra-high performance liquid chromatography coupled to low resolution tandem mass spectrometry and high resolution mass spectrometry are comparable, in terms of sensitivity and selectivity, for the determination of two kinds of veterinary drugs (nine glucocorticoids and five polyether ionophores) in a highly complicate matrices, such as influent and effluent sewage.

Highly confidence level of the results was achieved with MS/MS and HRMS methods for the determined compounds in analysed samples because 5.5 and 8 identification points were used for MS/MS and HRMS, respectively. However, the use of HRMS can help to prevent false results because data is acquired in full-scan mode so interferences coming from the matrix can be identified and evaluated. It has been shown that HRMS provides excellent confirmatory performances and quantitative capabilities.

Quality parameters (LODs, LOQs,

linear range and repeatability) with both methods show very good values and they were very similar between them. Nonetheless, LODs and LOQs for some polyether ionophores were slightly better with HRMS measurements. LODs and LOQs were always below to 10 ng L^{-1} and 50 ng L^{-1}, respectively, and repeatabilities (%RSD, n=3) were lower than 9%.

The correlation curve obtained representing the concentration measured for each compound in the same sample with both methods demonstrates that MS/MS and HRMS methods are comparable obtaining a determination factor of 0.996, an equation slope of 0.997 and a y-intercept point of 2.1.

Glucocorticoids were the compounds most frequently determined both in influent and effluent sewage samples being their concentration higher in influent than in effluent sewage. Nonetheless, the polyether ionophore monensin was also found in some sewage samples. Triamcinolone acetonide was only detected with UHPLC-HRMS method due to the better LOD achieved for this compound with this technique.

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3.2.4. Discussion of results

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To obtain the best separation of the five polyether ionophores, two different stationary phases (C_{18} and RP-amide) were tested. The best separation was achieved using the RP-amide column provided by Supelco® with fused-core technology, which provides fast chromatographic separations and efficacy similar to levels obtained by using UHPLC columns. With this column, and using a ternary mobile phase consisting of ultrapure water acidified with formic acid (0.1%) and a mixture of methanol and acetonitrile (1:1, v/v) as an organic modifier of the mobile phase, the complete chromatographic separation of the five compounds was obtained in less than 5 min. Moreover, both columns offered different selectivity for these compounds because different elution profiles were observed. This different selectivity is probably due to the numerous hydroxyl and ether groups present in polyether ionophores, which interact with the amide group embedded in the alkyl chain of the stationary phase. Although this column was designed to enhance the retention of polar compounds, it can offer different selectivity for non-polar compounds due to the selective interaction with oxo-groups [1,2].

In addition, different mass spectrometric conditions were tested to ascertain which option offers the best performance for obtaining the best LODs. To this end, positive and negative ionisation and ESI and APCI interfaces were tested. As reported in several studies focused on mass spectrometry of polyether ionophores, these compounds ionise as sodium adducts in positive mode due to their ability to complex alkaline metals. Other predominant ions were not observed [3]. However, the fragmentation of sodium adducts is often complicated and, for this reason, negative ionisation was also tested. However, neutral molecules are formed during ionisation because these compounds also wrap sodium in their structure if they are deprotonated. As mentioned before, both positive and negative APCI were tested but similar ionisation features to ESI were obtained. Moreover, the intensities obtained using APCI were much lower than ESI and, therefore, positive ESI was selected for the ionisation of polyether ionophores. As in previous studies, three SRM transitions were chosen in line with the guidelines of Commission Decision 2002/657/EC [4].

For the optimisation of the extraction method for water samples based on SPE, Oasis HLB and Oasis MAX sorbents were tested because the acidic properties of polyether ionophores. As expected due to the anionic exchange properties of Oasis MAX, better recoveries were obtained with this sorbent (64% to 103%) as compared to Oasis HLB (63% to 76%). However, the formation of neutral sodium complexes by adding sodium acetate to samples prior to SPE was tested in order to

enhance their retention on the Oasis HLB sorbent. This option resulted in an increase in recoveries because the neutral molecules are more retained by the Oasis HLB sorbent and recovery values between 88% and 110% were obtained.

For the extraction of polyether ionophores from sewage sludge samples using PLE, the best results were obtained with acetone or methanol. With respect to sample clean-up, of all the strategies tested, the ones which reduced the matrix effect most effectively were dilution of the matrix and modification of the elution gradient to enhance the separation of target analytes from matrix coeluting substances. However, SPE clean-up using Oasis HLB sorbent also offers a considerable reduction in the matrix effect for some compounds (e.g. salinomycin), as can be seen in Figure 3.2.1, which shows the apparent recoveries obtained with and without the SPE clean-up strategies tested. However, the apparent recoveries obtained were not enough in the case of all the compounds and so the SPE clean-up was discarded.

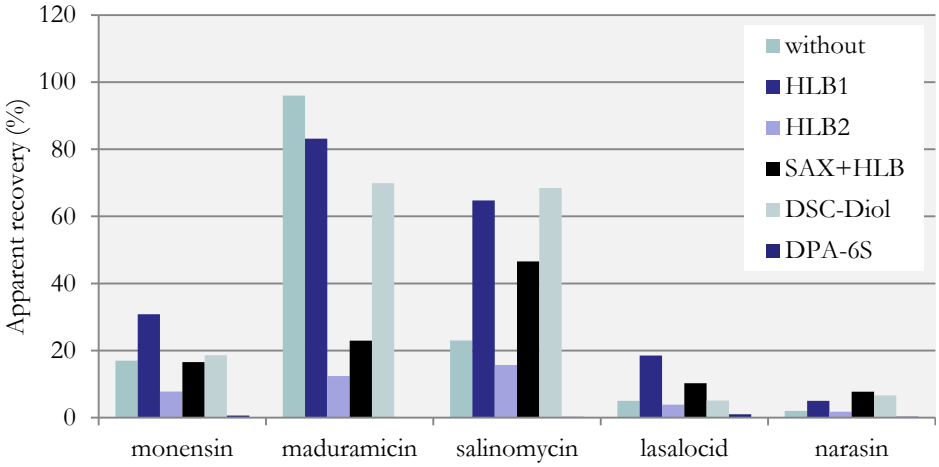

Figure 3.2.1. Apparent recoveries obtained for the five polyether ionophores using different sorbents or no sorbent (without) after PLE extraction. HLB1 refers to the elution step with pure methanol and HLB2 refers to the elution step with a mixture of methanol/methyl tert-butyl ether (9:1).

In the study which compares tandem mass spectrometry with high resolution mass spectrometry for the simultaneous determination of glucocorticoids and polyether ionophores in sewage samples. In this study, a method based on SPE followed by UHPLC was developed by combining the previously developed

methods for glucocorticoids and polyether ionophores. The SPE method used was the one developed for polyether ionophores because higher recoveries were obtained for both groups of compounds. As regards chromatographic separation, the C_{18} UHPLC column used for glucocorticoids was selected because it provides better separation for these compounds than the RP-amide column. The UHPLC method was then coupled either to QqQ operating in SRM mode or Orbitrap/Exactive with HRMS measurements. Both methods were applied to the analysis of sewage samples and excellent correlation with the determined concentrations was obtained by both instruments. Moreover, the concentrations determined are in accordance to those previously reported from different samples from the same STPs using the previously developed methods, which also demonstrate the applicability of the methods developed. Additionally, the confirmatory capabilities of both mass spectrometry measurements were evaluated in line with the guidelines of Commission Decision 2002/657/EC [4] and it has been observed that HRMS measurements are less susceptible to false negatives than SRM measurements, because a false negative for lasalocid was obtained when QqQ was used. This false negative was caused by an interfering compound on the confirmatory SRM transition of lasalocid.

As regards the samples analysed, polyether ionophores were not detected in samples from the rivers Ebre, Ter, Llobregat and Francolí. Samples from rivers as they pass through Catalonia have been previously analysed by other authors who did not find polyether ionophores in the samples either [5]. In the case of sewage samples, monensin and narasin were frequently determined in samples from the Reus STP but never in samples from the Tarragona STP (salinomycin was also determined in one sample from the Reus STP). This could be explained because the Reus STP is surrounded by more farming activities than the Tarragona STP and it has been reported that farming wastes could be one of the most important routes for the intrusion of these compounds into sewage treatment plants. Moreover, these two compounds were also frequently determined in sewage sludge from the same STP but, in this case, they are also found in some samples from the Tarragona STP, perhaps because they accumulate in sewage sludge during sewage treatment or maybe due to seasonal variations, as suggested by other authors [6].

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3.3. Determination of benzotriazoles, benzothiazoles and benzenesulfonamides in environmental samples

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The studies included here focus on the development of analytical methods for the determination of benzotriazole, benzothiazole and benzenesulfonamide derivates in environmental samples. These compounds are high production volume chemicals and were chosen because some of them have been shown to be toxic to aquatic species and 1-H-benzotriazole is a suspected human carcinogen [1-5].

One of the objectives of these studies was the development of analytical methods that allowed the simultaneous determination of these three families of compounds because this had only previously been achieved with GCxGC-TOF [6]. Thus, a liquid chromatography method for these compounds had not been proposed before our studies and this was the reason for attempting it here. As previously discussed in the introduction, there are several methods for determining benzotriazoles and benzothiazoles by LC, but methods for the determination of benzenesulfonamides are very scarce.

In line with previous sections, different chromatographic columns and SPE sorbents (Oasis HLB and MAX) were tested in the first study to find the best option for their simultaneous determination in aqueous environmental matrices. The second and third studies regard the development of two different extraction methods for sewage sludge. One of them focuses on QuEChERS extraction, which has never before been applied to sludge samples. However, at the same time as this study was being conducted, QuEChERS was also proposed for the determination of several pharmaceutical compounds in sewage sludge [7]. QuEChERS is used because of the great advantages which this extraction method can offer when applied to sewage sludge samples, including simplicity, fastness and low cost, which can be very useful for monitoring studies. The other extraction method proposed is based on pressurised hot water extraction (PHWE), because of the relatively high polarity of benzotriazoles, benzothiazoles and benzenesulfonamides and the previous experience of our research group with this environmentally friendly technique, which has been used for the determination of other EOCs. Moreover, the use of water as the extraction solvent also allowed an SPE clean-up to be performed directly after extraction, avoiding the time-consuming evaporation step that is required if organic solvents are used.

The developed methods were applied to determine these compounds in surface water samples from the rivers Ter, Llobregat, Ebre and Tordera, and sewage and sewage sludge samples from the STPs located in Tarragona, Reus, Vila-Seca/Salou, Blanes, Castell-Platja d'Aro and Palamós. Some of these STPs are equipped with

different tertiary treatments and these samples were also analysed to expand the available information on the removal of benzotriazole, benzothiazole and benzenesulfonamide compounds by tertiary treatments. For all of these STPs, data regarding the occurrence of benzotriazoles, benzothiazoles and benzenesulfonamides had never been reported before.

The results of these studies have been published in the *Journal of Chromatography A 1263 (2013) 7-13*, *Journal of Chromatography A 1339 (2014) 34-41* and *Journal of Chromatography A 1355 (2014) 53-60*.

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3.3.1. Efficient tandem solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry method to determine polar benzotriazole, benzothiazole and benzenesulfonamide contaminants in environmental water samples

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EFFICIENT TANDEM SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TRIPLE QUADRUPOLE MASS SPECTROMETRY METHOD TO DETERMINE POLAR BENZOTRIAZOLE, BENZOTHIAZOLE AND BENZENESULFONAMIDE CONTAMINANTS IN ENVIRONMENTAL WATER SAMPLES

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Abstract

An analytical method has been developed that allows the simultaneous determination of five benzotriazole (BTRs), four benzothiazole (BTs) and five benzenesulfonamide (BSAs) derivates. The method is based on tandem solid-phase extraction (SPE) with Oasis HLB followed by a clean-up step with Florisil. The chromatographic analysis was performed in less than 15 min and detection was carried out with a triple quadrupole mass analyser operating in *Multiple Reaction Monitoring* (MRM) mode. A comparison was performed between Oasis HLB and Oasis MAX sorbents for the solid-phase extraction, with Oasis HLB being the sorbent that gave the highest recoveries, ranging between 75% and 106%, depending on the compound and the matrix analysed. The proposed clean-up with Florisil sorbent reduced the matrix effect to below 20%. The repeatability (%RSD, 50-3,000 ng/L, n=3) of the method was less than 15% for all of the compounds in all of the matrices. The limits of detection (LODs) achieved ranged from 1 ng/L for BTR in river water up to 100 ng/L for BT in influent sewage. All of the compounds were determined in environmental waters such as river water and sewage. The highest concentrations determined corresponded to influent sewage samples in which the sum of concentrations for all compounds were between 4.6 μg/L and 8.0 μg/L. These concentrations were slightly reduced in secondary effluent and tertiary effluent sewage. Moreover, samples from tertiary effluent sewage based on ultra-filtration membrane treatments were also analysed and preliminary results seem to indicate that these treatments may be most effective for removing BTR, BT and BSA derivates.

Keywords: *Benzotriazole derivates; Benzothiazole derivates; Benzenesulfonamide derivates; Liquid chromatography-tandem mass spectrometry; Solid-phase extraction; Environmental waters.*

1 Introduction

Benzotriazole (BTRs), benzothiazole (BTs) and benzenesulfonamide (BSAs) derivates are high production chemicals with a very broad range of applications in industry and households. These contaminants are considered to be emerging contaminants, having been classified as toxic to aquatic organisms and bacteria with long-term adverse effects [1], [2]. In addition, benzotriazoles and benzothiazoles are highly soluble in water and resistant to biodegradation [3].

Benzotriazole derivates, such as tolyltriazole (4- and 5-methyl-1-H-benzotriazole) and xylyltriazole (5,6-dimethyl-1-H-benzotriazole), are mainly used as corrosion inhibitors in dishwasher detergents, aircraft de-icing fluids, automotive antifreeze formulations, industrial cooling systems, metal-cutting fluids, brake fluids and solid cooling lubricants, among others, and as antifoggants in photography and ultraviolet light stabilizers in plastics. There is also some evidence that these chemical compounds may be human carcinogens [4]. Recently, the drinking water guidelines of Australia have indicated a limit of 7 ng/L for tolyltriazole [5].

Benzothiazole derivates are mainly used as vulcanisation accelerators in the manufacture of rubber, as biocides in paper and leather manufactures, as corrosion inhibitors in antifreeze formulations and photosensitizers in photography [6].

Benzenesulfonamide is used in the synthesis of dyes, photochemicals and disinfectants.

Para-toluenesulfonamide (*p*-TSA) is the main hydrolysis product of the antimicrobial agent Chloramine-T. It is used to treat diseases in poultry, swine and fish and as disinfectant for surfaces or machines in food industry [7]. Moreover, *p*-TSA is used as a plasticiser, an intermediate product in the synthesis of pesticides and drugs and a fungicide in paints and coatings [8]. The German Federal Environment Agency (UBA) proves the toxicological relevance of *p*-TSA and recommends a maximum concentration of 0.3 μg/L in drinking water [8]. *Ortho*-toluenesulfonamide (*o*-TSA) is used in the production process of saccharin [9]. *N*-methyl-*para*-toluenesulfonamide and *N*-ethyl-*para*-toluenesulfonamide are used as plasticisers for polyamides, shellac, cellulose acetate or protein materials [10].

Benzotriazole and benzothiazole derivates are well-known aquatic contaminants and several papers have described their presence in surface and ground water [1,3,10-20] and in sewage [1,10,11,15-19,21-30]. Benzotriazole and tolyltriazole are ubiquitous water contaminants due to their occurrence in the aquatic environment [2] and they are frequently determined at levels between 1 and 100 μg/L. Moreover, it is well known that these contaminants are not completely removed in conventional sewage treatment plants (STPs) [1,10,16-19,22,24-29,31]. However, little is known about the occurrence of

benzenesulfonamide derivates in the aquatic environment [3,7-10,32] because they have not received the same attention as benzotriazoles and benzothiazoles.

Analytical strategies for extracting benzotriazole, benzothiazole and benzenesulfonamide derivates from aqueous samples are related to solid-phase extraction (SPE) using polymeric balanced polar/non-polar sorbents [3,10,15,17-19,27,31] but octadecylsilyl [29] and mixed-mode strong anion exchange [28] sorbents have also been used. In addition, dispersive liquid-liquid micro-extraction (DLLME) and stir bar sorptive extraction (SBSE) [23,33] have also been used as extraction techniques for these compounds.

Liquid chromatography (LC) [1,17,28], gas chromatography (GC) [15,22,23] and two-dimensional gas chromatography (GCxGC) [10,14] coupled to single (MS) and tandem mass spectrometry (MS/MS) or high resolution mass spectrometry (HRMS) [3,10,34] are the most appropriate analytical techniques for the determination of these contaminants. Nonetheless, a more accurate determination of these compounds is achieved when soft ionization sources are used, such as electrospray, because of their high polarity and low mass.

The aim of the present paper is to develop an analytical method for the simultaneous determination of five benzotriazole (including the two tolyltriazole isomers), four benzothiazole and five benzene-sulfonamide derivatives (including *orto*- and *para*-toluenesulfonamide isomers) in environmental water samples. This method is applied to several river waters and influent and effluent (secondary and tertiary) sewage samples from different STPs in Catalonia (Spain). Some of the STPs included in this study use two kinds of tertiary treatments, such as UV irradiation, chlorination disinfection and sand filtration or ultra-filtration membrane-based advanced sewage treatments. Therefore, the capabilities of these two treatments for the removal of benzotriazole, benzothiazole and benzenesulfonamide derivates were also investigated because of the incomplete removal of these contaminants with the conventional secondary sewage treatments.

2 Experimental

2.1 Reagents and standards

The chemical standards of five benzotriazole derivates: 1-H-benzotriazole (BTR), 4-methyl-1-H-benzotriazole (4TTR), 5-methyl-1-H-benzotriazole (5TTR), 5,6-dimethyl-1H-benzotriazole (XTR) and 5-chloro-1-H-benzotriazole (ClBTR); four benzothiazole derivates: benzothiazole (BT), 2-aminobenzothiazole (NH_2BT), 2-hydroxibenzothiazole (OHBT) and 2-(methylthio)benzothiazole (MeSBT); and five benzenesulfonamide derivates: benzenesulfonamide (BSA), *orto*-toluenesulfonamide (*o*-TSA), *para*-toluenesulfonamide (*p*-TSA), *N*-methyl-*para*-toluenesulfonamide (Me-

p-TSA), *N*-ethyl-*para*-toluenesulfonamide (Et-*p*-TSA) were purchased from Sigma-Aldrich (St. Louis, USA). Their chemical structures are summarised in Table 1. Stock solutions of individual standards at 1,000 mg/L were prepared in methanol and stored at -20 ºC. A mixed working solution of 10 mg/L was prepared weekly in methanol.
Ultrapure water was obtained using an ultrapure water purification system from Veolia waters (Sant Cugat del Vallés, Spain). Acetonitrile (ACN), methanol (MeOH) and methyl *tert*-butyl ether (MTBE) were of HPLC grade and supplied by Prolabo (VWR, Llinars del Vallès, Spain). Acetic acid (LC-MS grade), hydrochloric acid, sodium hydroxide and ammonium acetate were purchased from Sigma-Aldrich and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

Samples from rivers and sewage in Catalonia (Spain) were analysed. The river samples were taken from the Ebre, Ter, Llobregat and Tordera rivers which are the country's most important rivers. These rivers pass through several industrial sites, and Catalonia's biggest airport is located near to the mouth of Llobregat River.
Influent and effluent sewage were collected from six sewage treatment plants (STPs) located in Reus, Tarragona and Vila-seca/Salou in the south-east of the country, and in Castell-Platja d'Aro, Palamós and Blanes in the north-east. Two 24h composite samples were collected in different days for each treatment stage in each STP. The STPs receive urban sewage and industrial discharges from a population of between 100,000 and 200,000 inhabitants. The six STPs use activated sludge for biological treatment. The STPs at Castell-Platja d'Aro, Palamós and Blanes employ tertiary treatments based on UV irradiation, chlorination and sand filtration and the STP at Vila-seca/Salou employs a tertiary treatment based on ultra-filtration membranes.
All samples were collected using pre-cleaned amber glass bottles and were filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborough, UK). Typically, samples were analysed within three days of their collection (stored at 4 ºC) or they were frozen at -20 ºC until analysis.

2.3 LC-(ESI)MS/MS analysis

Chromatographic analysis was performed with an Agilent 1200 series rapid-resolution liquid chromatograph (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). The chromatographic column used was an Ascentis Express C_{18} (100 x 2.1 mm, 2.7 μm) from Supelco (Sigma-Aldrich) with fused-core technology. Ascentis Express RP-amide (100 x 2.1 mm, 2.7 μm) and Ascentis Express C_{18} (50 x 4.6 mm, 2.7 μm) columns from Supelco, a Zorbax

Table 1. Chemical structure, cone voltage (C.V.), collision energies (C.E.) and MRM transitions and their ion ratios of studied compounds.

Compound	Structure	C.V. (V)	Precursor ion (m/z)	C.E. (eV)	Product ions (m/z)	Ion ratio (%)
Benzotriazoles						
1H-benzotriazole (BTR)		100	120 $[M+H]^+$	25	**65** $[M+H\text{-}N_2\text{-}HCN]^+$	
				20	92 $[M+H\text{-}N_2]^+$	16
4-methyl-1H-benzotriazole (4TTR)		100	134 $[M+H]^+$	30	**77** $[M+H\text{-}N_2\text{-}HCN\text{-}H_2]^+$	
				20	79 $[M+H\text{-}N_2\text{-}HCN]^+$	46
				55	51 $[M+H\text{-}N_2\text{-}HCN\text{-}H_2\text{-}C_2H_2]^+$	33
5-methyl-1H-benzotriazole (5TTR)		100	134 $[M+H]^+$	30	**77** $[M+H\text{-}N_2\text{-}HCN\text{-}H_2]^+$	
				20	79 $[M+H\text{-}N_2\text{-}HCN]^+$	46
				55	51 $[M+H\text{-}N_2\text{-}HCN\text{-}H_2\text{-}C_2H_2]^+$	33
5,6-dimethyl-1H-benzotriazole (XTR)		100	148 $[M+H]^+$	30	**77** $[M+H\text{-}N_2\text{-}HCN\text{-}H_2\text{-}CH_2]^+$	
				20	91 $[M+H\text{-}N_2\text{-}HCN\text{-}H_2]^+$	40
				25	93 $[M+H\text{-}N_2\text{-}HCN]^+$	38
5-chloro-1H-benzotriazole (ClBTR)		100	154 $[M+H]^+$	25	**99** $[M+H\text{-}N_2\text{-}HCN]^+$	
				40	73 $[M+H\text{-}N_2\text{-}HCN\text{-}C_2H_2]^+$	97
				20	90 $[M+H\text{-}N_2\text{-}HCl]^+$	58

Table 1. (Cont.).

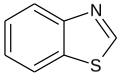
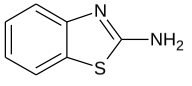
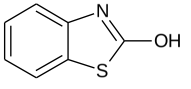
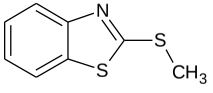
Compound	Structure	C.V. (V)	Precursor ion (m/z)	C.E. (eV)	Product ions (m/z)	Ion ratio (%)
Benzothiazoles						
Benzothiazole (BT)		70	136 $[M+H]^+$	40 25 77	**109** $[M+H\text{-}HCN]^+$ 65 $[M+H\text{-}HCN\text{-}CS]^+$ 77 $[M+H\text{-}HSCN]^+$	 99 31
2-aminobenzothiazole (NH_2BT)		100	151 $[M+H]^+$	30 40 25	**109** $[M+H\text{-}C(NH)_2]^+$ 65 $[M+H\text{-}C(NH)_2\text{-}CS]^+$ 124 $[M+H\text{-}HCN]^+$	 79 54
2-hydroxybenzothiazole (OHBT)		100	152 $[M+H]^+$	20 30 20	**119** $[M+H\text{-}HS]^+$ 124 $[M+H\text{-}CO]^+$ 92 $[M+H\text{-}CSO]^+$	 68 60
2-(methylthio)benzothiazole (MeSBT)		100	182 $[M+H]^+$	30 45 40	**167** $[M+H\text{-}^{\bullet}CH_3]^+$ 109 $[M+H\text{-}^{\bullet}CH_3\text{-}^{\bullet}NCS]^+$ 123 $[M+H\text{-}^{\bullet}CH_3\text{-}CS]^+$	 17 17

Table 1. (Cont.).

Compound	Structure	C.V. (V)	Precursor ion (m/z)	C.E. (eV)	Product ions (m/z)	Ion ratio (%)
Benzenesulfonamides						
Benzenesulfonamide (BSA)		70	156 $[M-H]^-$	15	**92** $[M-H-SO_2]^-$	
				25	64 $[M-H-{}^{\bullet}C_6H_6N]^-$	40
				55	79 $[M-H-{}^{\bullet}C_6H_5]^-$	36
para-toluenesulfonamide (*p*-TSA)		100	170 $[M-H]^-$	15	**106** $[M-H-SO_2]^-$	
				25	64 $[M-H-{}^{\bullet}C_7H_8N]^-$	34
				60	79 $[M-H-{}^{\bullet}C_7H_7]^-$	30
orto-toluenesulfonamide (*o*-TSA)		100	170 $[M-H]^-$	30	**79** $[M-H-{}^{\bullet}C_7H_7]^-$	
				65	64 $[M-H-{}^{\bullet}C_7H_8N]^-$	90
				10	106 $[M-H-SO_2]^-$	56
N-methyl-*p*-toluenesulfonamide (Me-*p*-TSA)		70	186 $[M+H]^+$	15	**91** $[M+H-NH_2CH_3-SO_2]^+$	
				5	155 $[M+H-NH_2CH_3]^+$	51
				45	65 $[M+H-{}^{\bullet}C_7H_7-NH_2CH_3]^+$	36
N-ethyl-*p*-toluenesulfonamide (Et-*p*-TSA)		70	200 $[M+H]^+$	15	**91** $[M+H-NH_2C_2H_5-SO_2]^+$	
				5	155 $[M+H-NH_2C_2H_5]^+$	56
				45	65 $[M+H-{}^{\bullet}C_7H_7-NH_2C_2H_5]^+$	40

Eclipse XDB-C_{18} (50 x 4.6 mm, 1.8 μm) column from Agilent and a Kinetex C_{18} (100 and 150 x 4.6 mm, 2.7 μm) column from Phenomenex (Le Pecq Cedex, France) were tested for the chromatographic optimisation.

The separation was performed in gradient elution with ultrapure water/ACN (98:2) 0.1% CH_3COOH (solvent A) and methanol (Solvent B) as the mobile phase. The gradient started isocratic at 0.5% B for 5.25 min and then increased to 18% in 3.5 min, remaining constant for a further 1.25 min, and up to 95% in 3 min. It then remained constant for 3 min. The oven temperature was maintained at 50 ºC and the flow rate was 0.8 mL/min. The injection volume was 20 μL. All of the compounds were eluted in less than 15 min.

Injections of individual standards, without a column, were used to optimise the conditions of the mass spectrometer. These conditions were as follows: nebulizer pressure of 45 psi, drying gas (N_2) flow rate of 11 L/min, drying gas temperature of 325 ºC, and capillary voltage of 3,000 V in positive mode and -4,500 V in negative mode.

The acquisition mode used was multiple reaction monitoring (MRM) and the cone voltage and collision energies were then optimised in order to select three characteristic MRM transitions for each compound (shown in Table 1) following the guidelines of European Directive 96/23/EC [35].

2.4 Solid-Phase Extraction

Sample volumes were 100 mL for STP influent, 250 mL for STP secondary effluent and 500 mL for STP tertiary effluent and river water. The SPE cartridges tested were Oasis HLB (150 mg, 6 cc) and Oasis MAX (150 mg, 6 cc) both from Waters (Wexford, Ireland). 6 cc SPE cartridges lab-packed with 500 mg of Florisil (bulk adsorbent from Sigma-Aldrich) were used to clean up the sample. A vacuum pump connected to a manifold (Teknokroma, Sant Cugat del Vallés, Spain) was used for the SPE procedure.

The Oasis HLB cartridge was preconditioned with 5 mL of MeOH followed by 5 mL of water prior to loading the samples into the cartridge at a flow-rate of ~10 mL/min. Before the elution step, 5 mL of ultrapure water was passed through the sorbent and, afterwards, it was dried under vacuum. The cartridge was then connected to the top of a Florisil cartridge (previously conditioned with 5 mL of MeOH) and the analytes were eluted from the Oasis HLB cartridge and passed through the Florisil cartridge with 2 x 3 mL of MeOH. The eluate was concentrated (100 μL, ca.) under a flow of nitrogen gas and made up to 1 mL with ultrapure water. The extract was filtered (0.22 μm PTFE) and injected into the LC-MS/MS system.

3 Results and discussion

3.1 Liquid chromatography-tandem mass spectrometry

The ionisation (positive or negative) of benzotriazole, benzothiazole and benzenesulfonamide derivates was studied by direct injection into the MS/MS spectrometer of individual standard solutions of 1 mg/L with a mobile phase composition of 50:50 of solvent A and B. All of the compounds studied were ionised in positive mode as $[M+H]^+$ and 2-hydroxybenzothiazole, benzene-sulfonamide and *orto*- and *para*-toluenesulfonamide were also ionised in negative mode as $[M-H]^-$. However, the response for 2-hydroxybenzothiazole was higher in positive mode and for benzenesulfonamide and *orto*- and *para*-toluenesulfonamide, the response was higher in negative mode under the aforementioned conditions. Subsequently, the source parameters that affect the performance of the ESI interface were individually optimised for each compound. The optimised parameters and the ranges tested were: nebuliser pressure (20 to 60 psi), drying gas temperature (150 ºC to 350 ºC), drying gas flow (5 to 13 L/min) and capillary voltage (1,500 to 6,000 V). The values that provided the best response were selected as a compromise between the optimal values for each compound and are described in Section 2.3. Cone voltage (0 to 200 V) and collision energies (5 to 70 eV) for the fragmentation of the precursor ions of these compounds were also optimised individually in order to obtain three MRM transitions for each compound, except for 1-H-benzotriazole, which only generates two product ions because of its structure and low mass. The proposed structures for product ions obtained and their respective cone voltage and collision energies are shown in Table 1.

Benzotriazole derivates have common fragmentations pathways based on the loss of one nitrogen molecule (N_2) and one hydrogen cyanide molecule (HCN), resulting in the most intense mass fragments. The fragmentation pathways of benzothiazole derivates are more closely linked to the group labelled in position two (carbon) of the heterocyclic ring. Therefore, the losses of this group together with the carbon are specific for each compound, such as the loss of carbon monoxide for 2-hydroxybenzothiazole. Moreover, the loss of hydrogen cyanide molecule (HCN) is also common for all of the benzothiazole derivates. The formation of a hydrogen adduct precursor for benzothiazole and 2-(methylthio)benzothiazole is less intense than the other two substituted benzothiazole derivates, resulting in less intense MRM transitions for these two compounds and therefore, in higher LODs and LOQs for these two compounds. Benzothiazole gives two MRM transitions with equal intensity but the transition 136>109 was chosen for quantification due to their lower chemical noise than the transition 136>65.

Benzenesulfonamides derivates are fragmented by the sulfonamide group, resulting in the loss of the radicalary benzene molecule and/or

the loss of the sulphur dioxide molecule (SO_2). For the two toluenesulfonamide isomers, their fragmentation was the same but the most intense transitions were different, with the transition 170>106 being the most intense for *para*-toluenesulfonamide and 170>79 for *orto*-toluenesulfonamide. This fact is important because it enhances selectivity in the mass spectrometric detection for the toluenesulfonamide isomers.

Because of the high polarity of these compounds and their similar chemical structures, which make their chromatographic separation difficult, several parameters were studied including different octadecylsilyl columns and one polar-embedded reversed-phase column. Firstly, the chromatographic separation was optimised with an Ascentis Express C_{18} column (2.1 x 100 mm, 2.7 μm) and 0.1% of CH_3COOH in water and ACN as mobile phases at 30 ºC. In order to perform the separation of the two isomers pairs (TTRs and TSAs), it was necessary to start the gradient at a very low composition of organic mobile phase. This composition was kept low until the elution of the two pairs of isomers. The gradient could then be increased quickly to reduce the analysis time without compromising the separation of the other compounds.

Next, the chromatographic method was transferred to an Ascentis Express RP-amide column (2.1 x 100 mm, 2.7 μm) in order to compare the different selectivity of these two stationary phases. The retention factor for these compounds was similar between the two columns but the elution profile changed for some compounds. Thus, BT, ClBTR, OHBT and Me-*p*-TSA change their elution order. However, the RP-amide column did not provide any separation of the isomers *o*-TSA/*p*-TSA and 4TTR/5TTR. For this reason the RP-amide column was discarded for further optimisation.

Aqueous solutions at different pHs (3, 5 and 7) were prepared with CH_3COONH_4 and/or CH_3COOH to study the effect of pH in the separation. These solutions were tested with an Ascentis Express C_{18} column but pH only affected the retention factor of NH_2BT due to the pK_a of the amino group (~7.8). As the chromatographic separation of NH_2BT is not complicated under any of the pHs tested, and taking into account that the response in the MS/MS of these studied compounds was higher when ultrapure water with 0.1% of CH_3COOH was used as the aqueous mobile phase instead of the other solutions tested (which contained CH_3COONH_4), ultrapure water with 0.1% of CH_3COOH (pH of 3.2) was selected.

Moreover, methanol and acetonitrile were tested as organic components of the mobile phase and it was observed that this parameter did not modify the selectivity of the column but it did affect the separation of isomers. Methanol provided better separation, although not complete, for the 4TTR/5TTR couple and acetonitrile for the *o*-TSA/*p*-TSA, which were not separated with methanol. To improve

the separation of these isomers, different portions of acetonitrile (2%, 5% and 10%) were added to aqueous mobile phase as *o*-TSA and *p*-TSA were eluted before TTR isomers and their separation is linked with the use of acetonitrile. The best separation of the two pairs of isomers was obtained with a proportion of 2% of ACN in aqueous mobile phase with methanol as the organic mobile phase.

In addition, different sizes, kinds of particle and brands of octadecylsilyl columns (described in Section 2.3.) were tested. However, the best separation of the isomers was achieved with the Ascentis Express C_{18} column (2.1 x 100 mm, 2.7 μm). As the changes in the column used did not allow the complete separation of the isomers, an increase in the oven temperature was tested. This parameter improved the separation of the 4TTR/5TTR couple under the conditions mentioned above, with 50 ºC being the temperature that provided the best separation for the isomers and a complete separation between the other compounds studied, in less than 14 min.

3.2 Solid-phase extraction

Because of the high polarity of benzotriazole, benzothiazole and benzenesulfonamide derivates, and based on previous papers published [3,10,15,17-19,27,31], the most suitable option for extracting these compounds from aqueous matrices is polymeric balanced polar/non-polar sorbents (Oasis HLB in our case). However, a paper by Carpinteiro *et al.* [28] on the determination of benzotriazole and benzothiazole derivates using mixed-mode strong anion exchange sorbent (Oasis MAX) has recently been published, reporting recoveries of over 80% for the majority of the compounds studied. One of the benefits of the use of mixed-mode strong anion exchange sorbents in comparison to conventional polymeric balanced polar/non-polar sorbents is the theoretically reduction of the matrix effect in the electrospray interface when environmental water samples are analysed by LC-MS methods. This reduction in the matrix effect is performed by the selective retention of interfering substances, such as humic and fulvic acids, for example, into the sorbent under specific elution conditions.

In the present study, these two kinds of sorbents were evaluated because information regarding the usage of Oasis MAX is limited to the paper mentioned above [28] and its applicability for the extraction of benzenesulfonamides from environmental waters has not previously been reported. Therefore, Oasis HLB (150 mg) and Oasis MAX (150 mg) were tested with standard solutions (100 mL of ultrapure water spiked at 2.5 μg/L) in order to evaluate their suitability. For Oasis HLB, three pHs (3, 7 and 10) were tested, adjusted with hydrochloric acid or sodium hydroxide solutions, and for Oasis MAX, only pH 10 was tested in order to deprotonate the compounds to enhance their retention into the sorbent by ion exchange interactions.

The elution of Oasis HLB was performed twice with 3 mL of methanol each time or with a mixture of methyl *tert*-butyl ether and methanol (9:1) because the MTBE/MeOH mixture is not capable of eluting humic substances from Oasis HLB sorbent. These substances are present in environmental waters and can cause a matrix effect. In the case of Oasis MAX, the elution was performed in two steps. The first elution is the same as for Oasis HLB (two times with 3 mL of methanol) and the second elution step was conducted twice with 3 mL of acidified methanol (5% of CH_3COOH) to elute the compounds retained with an ion exchange mechanism. The procedural steps not mentioned are the same as those described in Section 2.4.

Table 2. Recoveries (%) obtained for 250 mL of spiked effluent sewage (1 μg/L) with Oasis HLB at three pHs and with Oasis MAX. For conditions, see text.

Compound	**Oasis HLB**			**Oasis MAX**
pH	**3**	**7**	**10**	**10**
BSA	121	86	109	60
NH_2BT	95	109	84	64
BTR	70	107	77	64
o-TSA	111	89	95	73
p-TSA	119	91	102	77
4TTR	98	100	90	51
5TTR	96	104	88	47
ClBTR	90	92	85	23
OHBT	103	99	98	32
BT	115	88	60	45
Me-*p*-TSA	105	99	103	76
XTR	88	94	90	21
Et-*p*-TSA	115	100	111	74
MeSBT	93	98	33	12

%RSD(n=3)<15%.

In the first instance, the elution with MTBE/MeOH was discarded because the recoveries obtained with Oasis HLB (around 50%) were much lower than those obtained under the same conditions with pure methanol (around 90%). With regard to the pH of sample, no significant differences in the recovery values with Oasis HLB sorbent were observed at the three pHs tested. Comparing these results with the recoveries obtained when combining the two elution steps in Oasis MAX sorbent, lower recoveries were obtained when Oasis MAX was used (between 70% and 90%). If the two elution steps are analysed separately, it is observed that

BSA, NH_2BT, *o*-TSA, *p*-TSA, Me-*p*-TSA, BT, Et-*p*-TSA and MeSBT were eluted with pure methanol which indicates that these compounds were not deprotonated at pH 10 and, therefore, they do not interact by an ion exchange mechanism. BTR and ClBTR were only eluted with acidified methanol and the compounds 4TTR, 5 TTR, OHBT and XTR were partially eluted with the two elution steps, which indicates that their pKa is close to 10. Therefore, it is necessary to elute the

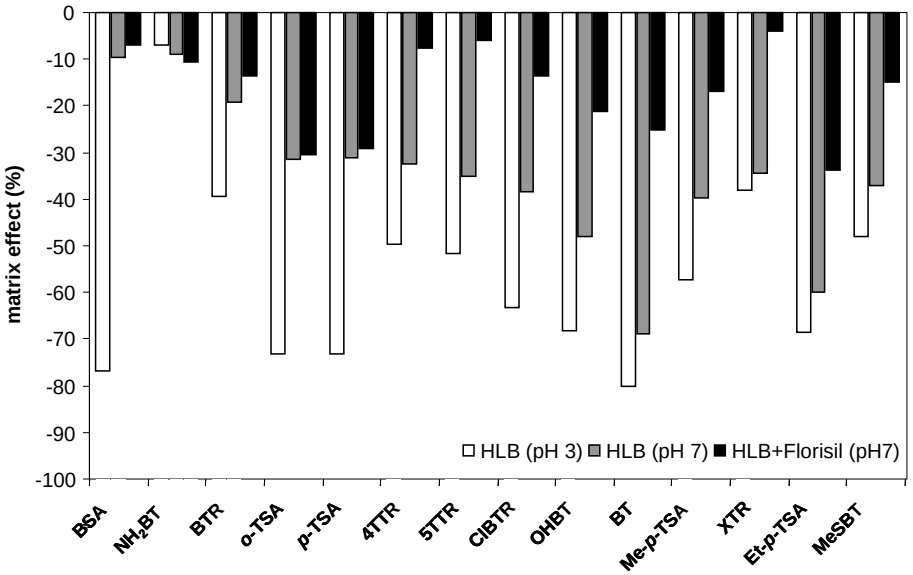


Fig. 1. Matrix effect (%) for each compound with Oasis HLB depending on the pH and the clean-up strategy developed.

compounds with acidified methanol in a single step to determine all of the compounds simultaneously with Oasis MAX sorbent.

Both sorbents were also checked to evaluate the recovery yield and matrix effect by analysing effluent sewage samples. Then, 250 mL of effluent sewage spiked at 1 μg/L was loaded into Oasis HLB (at pH 3, 7 and 10) and Oasis MAX (at pH 10) sorbent under the same conditions mentioned above. A non-spiked sample was also analysed to subtract the amount of compounds present in the sample. The recoveries are shown in Table 2 and they were calculated comparing the responses obtained for samples spiked before and after the SPE step. The recoveries obtained were similar to those obtained with ultrapure water for Oasis HLB, up to 90% for the majority of the compounds at pH 3 and 7. However, the recoveries at pH 10 for Oasis HLB and Oasis MAX were lower than in ultrapure water. Moreover, the sample at pH 10 flocculates (probably due to the high content of dissolved organic matter in sewage) and this fact significantly

complicates its passage through to the sorbent. Thus, pH 10 for Oasis HLB and Oasis MAX was discarded. To calculate the matrix effect, the responses obtained for samples spiked after SPE were compared with a standard solution at the same concentration ($(A_{sample}-A_{standard})\bullet 100/A_{standard}$). If the matrix effect is compared at pH 3 and 7, the ion suppression at pH 3 is higher than for pH 7 for the majority of the compounds, as shown in Figure 1. For this reason, the sorbent selected was Oasis HLB with a pH of 7 for loading the sample.

In order to reduce the matrix effect, a clean-up step after elution of the Oasis HLB cartridge was tested with a lab-packed Florisil SPE cartridge (500 mg), connecting the Florisil cartridge to the bottom of the Oasis HLB cartridge during the elution step. The methanolic eluate then passed through the Florisil sorbent (retaining interfering substances) and was collected for evaporation and injection into the LC-MS/MS system. Figure 1 also shows the results of this clean-up process for the same effluent sample mentioned above.

These extracts are much cleaner, resulting in less matrix effect (from between -30% and -60% to less than -20% for the most of the compounds) without affecting the recovery yield, as will be shown later. Therefore, this clean-up strategy was adopted.

Table 3. Recoveries (*R*, %) and matrix effect (*ME*, %) obtained for spiked samples with Oasis OASIS HLB in tandem with Florisil SPE.

Compound	River[a]		Influent[b]		Secondary effluent[c]		Tertiary effluent[a]	
	R	***ME***	***R***	***ME***	***R***	***ME***	***R***	***ME***
BSA	75	-5	97	-19	94	-7	79	-2
NH_2BT	88	3	87	4	101	-11	97	-16
BTR	93	1	91	-22	106	-13	103	8
o-TSA	94	-7	92	-24	101	-31	100	-2
p-TSA	92	-6	95	-22	99	-29	103	-6
4TTR	91	2	91	-6	100	-8	100	10
5TTR	93	1	95	-7	102	-6	105	6
ClBTR	85	-6	85	4	97	-14	98	5
OHBT	94	-14	96	-23	105	-21	102	9
BT	95	-19	100	-29	99	-25	94	-12
Me-*p*-TSA	93	-20	89	-12	101	-17	104	11
XTR	90	-12	92	1	95	-4	102	2
Et-*p*-TSA	93	-9	96	-25	100	-34	103	5
MeSBT	97	-9	100	-10	108	-15	107	-4

[a] 0.5 μg/L; [b] 2.5 μg/L; [c] 1 μg/L.
%RSD(n=3)<15%.

The optimised SPE extraction (Oasis HLB, pH 7) and clean-up step (Florisil) were tested for different spiked matrices in order to evaluate their benefits and check the sample volume in order to establish the definitive procedure. Sample volumes of environmental waters were tested and selected based on our previous experience and results, with 500 mL being selected for river water and tertiary effluent sewage samples, 250 mL for secondary effluent sewage samples and 100 mL for influent sewage samples. Thus, 500 mL of spiked river water (0.5 μg/L), 500 mL of spiked tertiary effluent sewage (0.5 μg/L), 250 mL of spiked secondary effluent sewage (1 μg/L) and 100 mL of spiked influent sewage (2.5 μg/L) were analysed to calculate the recoveries and matrix effect obtained. The recoveries (Table 3) were very similar for all matrices, with values between 75% (BSA) and 108% (MeSBT), depending on the compound and matrix. The recoveries for BSA were lower in river water and tertiary effluent than in influent and effluent sewage, due to the sample volume loaded and the fact that BSA is the most polar compound. The matrix effect observed (Table 3) was very low compared with the values obtained without clean-up (data not shown), which were usually higher than 30% for all of the compounds. Thus, the matrix effect was between 3% (NH_2BT) and -20% (Me-*p*-TSA) for river water, between 10% (4TTR) and -16% (NH_2BT) for tertiary effluent sewage, between -4% (XTR) and -34% (Et-*p*-TSA) for secondary effluent sewage and between 4% (4TTR) and -29% (BT) for influent sewage.

3.3 Method validation

These compounds are present in all of the samples analysed at relatively higher values than most of the emerging contaminants in environmental waters (μg/L levels). Therefore, it is not possible to use a matrix-matched calibration curve and a standard addition calibration is very time-consuming. As the matrix effect involved in the method developed is normally less than ±20% and the recoveries are higher than 90%, an external calibration curve is proposed for their quantification. It is possible to use an isotopic-labelled internal standard because d_4-BTR, $^{13}C_6$-BSA and *p*-^{15}N-TSA are commercially available (from Sigma-Aldrich) but all of them were eluted at the begin of the chromatogram and their usage may not be the most appropriate for all of the compounds.

The linear range, limits of quantification (LOQ) and detection (LOD), apparent recoveries (at two concentration levels) and repeatability (expressed as relative standard deviation (%RSD, n=3)) were calculated with an external calibration curve for all of the compounds under study. The recoveries and repeatability were evaluated in river water and secondary effluent sewage samples. These parameters are shown in Table 4. LOQs were calculated as the lowest point of the calibration

curve and LODs correspond to a signal more than three times the signal/noise ratio. Thus, LOQs and LODs were expressed as the amount (pg) of analyte injected (injection volume was 20 μL) and were from 20 pg_{inj} to 1000 pg_{inj} and from 10 pg_{inj} to 200 pg_{inj}, depending on the compound. The method was almost linear up to 20,000 pg_{inj}. The LODs and LOQs for each sample matrix were estimated from instrumental parameters because these compounds are ubiquitous water contaminants, with LODs between 1 ng/L for BTR and 20 ng/L for BT and LOQs between 2 ng/L for BTR and 100 ng/L for BT in river water or tertiary effluent sewage, for example. These limits were better than or equal to those reported previously in several papers for SPE/LC-MS methods [1,3,7,17,18,28]. In these papers, the authors are in agreement with respect to the estimation of the method detection limits from the instrumental detection limits, due to the facts mentioned above. As an example, Jover *et al.* [10], determine simultaneously the three families of compounds using GCxGC-TOF reported LODs between 10 ng/L (BT) and 112 ng/L (TTR) in sewage depending on the compound but these limits are calculated as the content of the blank plus three times its standard deviation. Using LC-MS/MS, Carpinteiro *et al.* [28], reported LOQs from 2 ng/L to 4 ng/L for river water, from 110 ng/L to 222 ng/L for effluent sewage and from 13 ng/L to 286 ng/L for influent sewage for some benzotriazoles and benzothiazoles derivates. Richter *et al.* [7] reported LOQs for *p*-TSA (10 ng/L), *o*-TSA (20 ng/L) and BSA (20 ng/L) calculated from purified water. As can be observed, the values mentioned above are similar or better than those reported in the present paper.

Apparent recoveries (R_{ap}), which include the recovery yield (R) and matrix effect (ME), are shown in Table 4 and were obtained from spiked river water (50 and 500 ng/L, 500 and 1,500 ng/L for BT and MeSBT) and effluent sewage samples (100 ng/L and 1,000 ng/L, 1,000 and 3,000 ng/L for BT and MeSBT). In addition, non-spiked samples were also analysed in order to subtract the response caused by compounds present in the samples and calculate the apparent recoveries. The apparent recoveries of benzotriazole, benzothiazole and benzene-sulfonamide derivates ranged from 69% (BSA) to 94% (BTR and 5TTR) for river water and from 70% (BT) to 96% (5TTR) for effluent sewage. To calculate the repeatability (%RSD), the analyses were carried out in triplicate, giving a result of less than 15% at the low spiked level for river water and sewage and less than 12% at the high spiked level. Therefore, these results demonstrate the potential application of the developed method for the analysis of several matrices of environmental waters.

Table 4. Retention time (t_R), limit of detection (LOD) and quantification (LOQ), apparent recovery (R_{ap}, %) and repeatability (%RSD, n=3) of the developed method for studied compounds.

Compound	t_R (min)	LOD (pg_{inj})	LOQ (pg_{inj})	River				Secondary effluent			
				R_{ap} [a] (%)	RSD (%)	R_{ap} [b] (%)	RSD (%)	R_{ap} [c] (%)	RSD (%)	R_{ap} [d] (%)	RSD (%)
BSA	1.7	20	50	69	13	71	8	82	10	88	6
NH_2BT	2.5	10	20	81	10	91	3	86	13	90	4
BTR	3.1	10	20	80	7	94	4	87	14	92	8
o-TSA	5.3	50	100	81	12	88	3	75	6	70	2
p-TSA	5.5	50	100	82	15	86	2	68	11	70	1
4TTR	9.2	10	20	84	12	93	7	87	10	92	6
5TTR	9.4	10	20	81	12	94	3	87	11	96	2
ClBTR	10.2	10	20	76	8	80	3	78	11	84	4
OHBT	10.6	20	50	73	7	81	4	75	11	82	6
BT	11.1	200	1000	72	12	77	10	70	4	74	8
Me-*p*-TSA	11.5	10	20	66	10	75	2	79	8	84	2
XTR	12.4	10	20	71	10	79	8	82	6	92	4
Et-*p*-TSA	12.8	10	20	79	11	85	10	64	8	66	7
MeSBT	13.7	100	500	77	6	88	5	71	12	92	12

[a] Samples spiked at 50 ng/L, except for BT and MeSBT which are spiked at 500 ng/L.
[b] Samples spiked at 500 ng/L, except for BT and MeSBT which are spiked at 1,500 ng/L.
[c] Samples spiked at 100 ng/L, except for BT and MeSBT which are spiked at 1,000 ng/L.
[b] Samples spiked at 1,000 ng/L, except for BT and MeSBT which are spiked at 3,000 ng/L.

4 Application to environmental samples

The method developed was applied for the determination of the five benzotriazole derivates, the four benzothiazole derivates and the five benzenesulfonamide derivates in several samples from four rivers and six STPs.

The concentrations found for sewage samples reported in this paper were the average concentration between two different samples for each treatment taken in different days. The presence of the compounds detected was confirmed with their ion ratios (Table 1), in accordance with the guidelines of European Directive 2002/657/EC [35]. An MRM chromatogram of tertiary effluent sewage sample is shown in Figure 2.

For river samples (Table 5), all of the compounds studied were determined, with the exception of Me-*p*-TSA and MeSBT, which were not positively detected, and BT and XTR, which were detected but not quantified in any river sample. The Ter and Llobregat Rivers had the highest concentration of these contaminants, but the Tordera River showed the highest value for 5TTR, with a concentration of 270 ng/L, while the Ebre River showed a higher concentration of *p*-TSA and NH_2BT than the other rivers investigated. Taking into account the total amount of these contaminants in river water, their values were between 300 ng/L in the Ebre River and 546 ng/L in the Tordera River.

With regard to sewage samples from the STPs under study, influent samples from STPs displayed a very high concentration of certain benzotriazole derivates (Tables 6 and 7). BTR was found at a concentration higher than 5,000 ng/L in one sample from the Reus STP, and TTR isomers were found at a concentration higher than 2,000 ng/L in some samples from the STPs in Reus, Tarragona, Castell-Platja d'Aro and Blanes. The other contaminants were present with concentrations between <10 ng/L and 500 ng/L.

All of these contaminants were also determined in secondary effluent sewage samples at concentrations slightly lower than influent samples. Once again, BTR and TTR isomers showed the highest concentration values. These compounds were found at levels higher than 1,000 ng/L in several samples and it should be noted that the concentration of *p*-TSA in these samples was higher than 200 ng/L. The other compounds were determined at levels between <5 ng/L and 120 ng/L.

The results of sewage samples collected after tertiary treatment can be distinguished between tertiary treatments based on chlorination, sand filtration and UV irradiation and tertiary treatments based on an ultra-filtration membrane. The former treatments are not capable of removing these contaminants, as shown in Table 7, where the results for these STPs are summarised.

The total contents included in Tables 6 and 7 are calculated by assigning an estimated (average) concentration value between their LOQ and LOD

Table 5. Concentration found (ng/L) of BTR, BT and BSA derivates investigated in river water samples.

Compound	River			
	Ebre	**Llobregat**	**Ter**	**Tordera**
BSA	9	6	11	10
NH_2BT	25	5	5	4
BTR	37	119	153	33
o-TSA	17	8	18	20
p-TSA	69	45	27	33
4TTR	26	49	46	69
5TTR	43	112	118	270
ClBTR	2	2	2	15
OHBT	8	32	45	6
BT	60	60	n.d.	60
Me-*p*-TSA	n.d.	n.d.	n.d.	n.d.
XTR	2	2	2	2
Et-*p*-TSA	3	2	2	25
MeSBT	n.d.	n.d.	n.d.	n.d.
Total	**300**	**441**	**429**	**546**

for the compounds detected at a level below their LOQ. As can be seen in the table, the removal of these contaminants is low in the STPs that applied the former tertiary treatments, with the total concentration for these contaminants found to be between 1,861 ng/L in the Castell-Platja d'Aro STP and 3,216 ng/L in the Palamós STP. However, when the tertiary effluent sewage samples from the ultra-filtration treatment at the Vila-seca/Salou STP were analysed (Table 6), the concentration of benzotriazole, benzothiazole and benzenesulfonamide derivates were strongly reduced, down to 60 ng/L for benzotriazole and down to 30 ng/L, approximately, for tolyltriazole isomers and benzothiazole. Benzenesulfonamide and toluene-sulfonamide isomers were not detected in these samples but Me-p-TSA and Et-p-TSA were identified. Therefore, these preliminary results obtained show that the tertiary treatment based on membrane ultra-filtration seems be more effective at removing these compounds from sewage than the other tertiary treatments included in this study.

The improvement in terms of benzotriazole removal with membrane-based treatments were previously reported by Weiss *et al.*, [24] with a lab-scale membrane bioreactor (MBR) and a pilot-scale ozonation plant for benzotriazole and tolyltriazole, compared with conventional sewage treatments at a municipal STP in Berlin. The pilot-scale ozonation treatment seems to be highly effective at removing benzotriazole, with efficiencies higher

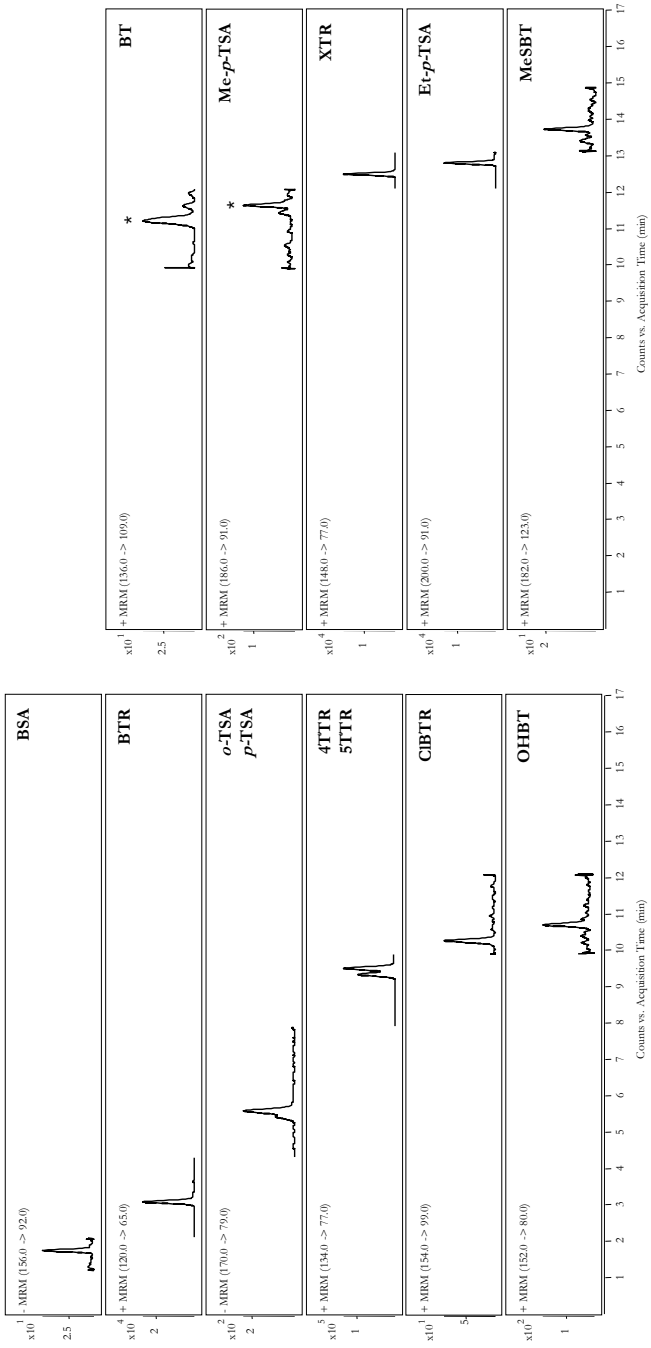


Fig. 2. MRM chromatogram of a tertiary effluent sample from Castell-Platja d'Aro STP.

than 99% with 1 mg O_3/mg DOC_0 (Dissolved Organic Carbon). However, the removal of these contaminants with MBR (between 14% for 4TTR and 61% for BTR) is less effective based on the results published. In the present study, an efficiency of removal with ultra-filtration membrane treatment higher than 80% can be expected from the preliminary results obtained for BTR, 4TTR and 5TTR.

The environmental concentration of these contaminants is very dependent on the emission source because their concentrations ranged from below one hundred ng/L to several thousand ng/L, depending on the compound and the matrix, demonstrating that these compounds are ubiquitous aqueous contaminants, as can be observed from the results obtained. Jover *et al.* [10] report an estimated concentration value of 17,153 ng/L for 4TTR in industrial STP effluent sewage in Catalonia. In contrast, this compound was not detected in urban STP effluent sewage [10]. Carpinteiro *et al.* [28] found concentrations higher than 100,000 ng/L of BTR and TTR isomers in industrial raw wastewaters from the north-west of Spain which receive discharges from metal industries. In a European study (including the north-east Spain region) about polar contaminants in the water cycle, Reemtsa *et al.* [25] reported values close to 1,000 ng/L and almost 500 ng/L for benzotriazole and benzothiazole derivates in effluent STP sewage and surface water, respectively. In addition, more studies at different sites report similar concentrations for benzotriazole and benzothiazole derivates in sewage samples [22] and in rivers [29]. However, most of these papers are limited to the determination of BTR, TTR, BT and MeSBT.

There has been less research into benzenesulfonamide derivates than into benzotriazole and benzothiazole derivates. However, Richter *et al.* [7,8] investigated the presence of these compounds in environmental waters in Berlin (Germany). They reported concentrations of around 5,000 ng/L for *p*-TSA, 400 ng/L for *o*-TSA, and 100 ng/L for BSA in STP influent sewage and around 1000 ng/L for *p*-TSA and *o*-TSA and 400 ng/L for BSA in STP effluent sewage. These concentrations are much higher than those reported in the present paper due to the differences in receiving flow mass in STPs studied. However, these results also confirm that *p*-TSA is the most significant benzenesulfonamide derivate found in sewage and it was not eliminated during sewage treatments.

Moreover, Jover *et al.* [10], who determined (*ortho*-, *meta*- and *para*-) ethyl toluenesulfonamide derivates simultaneously with benzotriazole and benzothiazole derivates, found *p*-TSA and Et-*p*-TSA at 256 ng/L and 155 ng/L, respectively, in the Besòs River. These compounds were also determined at levels of 94 ng/L and 831 ng/L in industrial effluent sewage. To the best of our knowledge, the presence of Me-*p*-TSA in the samples analysed has been

investigated for the first time in the present paper. This contaminant was not detected in the river samples analysed but it was determined in all of the sewage samples analysed, including secondary and tertiary effluent sewage.

Table 6. Concentration found (ng/L) of BTRs, BTs and BSAs investigated in south-east region STPs sewage samples.

Compound	Reus STP		Tarragona STP		Vila-Seca/Salou STP	
	Influent	**Secondary effluent**	**Influent**	**Secondary effluent**	**Secondary effluent**	**Tertiary effluent**
BSA	<LOQ	28	55	30	29	n.d.
NH_2BT	n.d.	8	10	14	9	n.d.
BTR	2994	1559	2212	1440	372	62
o-TSA	45	69	88	70	50	n.d.
p-TSA	157	238	352	143	173	n.d.
4TTR	2149	541	392	641	587	38
5TTR	2042	1485	1466	1037	655	22
ClBTR	11	5	<LOQ	4	4	n.d.
OHBT	172	9	199	45	14	5
BT	n.d.	n.d.	<LOQ	<LOQ	<LOQ	<LOQ
Me-*p*-TSA	<LOQ	4	<LOQ	4	4	2
XTR	<LOQ	16	<LOQ	3	4	2
Et-*p*-TSA	36	30	59	40	26	2
MeSBT	321	<LOQ	<LOQ	<LOQ	n.d.	<LOQ
Total	**7955**	**4053**	**5308**	**3591**	**1989**	**182**

5 Conclusions

An analytical method has been developed based on tandem solid-phase extraction followed by liquid chromatography-triple quadrupole mass spectrometry for the simultaneous determination of five benzotriazole, four benzothiazole and five benzenesulfonamide derivates. The chromatographic separation of the two tolyltriazole and the two toluenesulfonamide isomers pairs was achieved and therefore, their individual determination and quantification was possible.

Recoveries obtained with Oasis HLB in tandem with a Florisil SPE cartridge gave values between 75% and 106%, depending on the compound and the matrix analysed, with a matrix effect of less than 20%. These two facts allow their quantification with an external calibration curve. The repeatability (%RSD, 50-3,000 ng/L, n=3) of the method was less than 15% for all of the compounds in all of the matrices.
When the method was applied for the determination of these compounds in sewage and river water samples, all of them were determined at

Table 7. Concentration found (ng/L) of BTRs, BTs and BSAs investigated in north-east region STPs sewage samples.

	Castell-Platja d'Aro STP		**Palamós STP**		**Blanes STP**			
Compound	**Influent**	**Tertiary effluent**	**Influent**	**Secondary effluent**	**Tertiary effluent**	**Influent**	**Secondary effluent**	**Tertiary effluent**
BSA	<LOQ	9	<LOQ	21	13	26	45	30
NH_2BT	<LOQ	n.d.	23	19	n.d.	11	12	n.d.
BTR	562	148	727	325	317	543	222	151
o-TSA	44	31	38	49	42	48	78	80
p-TSA	178	177	187	255	233	245	372	428
4TTR	1859	615	1665	1253	1043	2062	849	1067
5TTR	1544	717	1440	1538	1386	1797	1363	1535
ClBTR	<LOQ	<LOQ	<LOQ	4	5	15	5	5
OHBT	136	7	134	18	12	260	111	87
BT	<LOQ	<LOQ	<LOQ	n.d.	<LOQ	<LOQ	120	<LOQ
Me-*p*-TSA	<LOQ	<LOQ	<LOQ	4	2	<LOQ	5	3
XTR	<LOQ	27	11	70	38	<LOQ	6	4
Et-*p*-TSA	37	36	61	74	49	70	44	43
MeSBT	<LOQ	<LOQ	<LOQ	60	<LOQ	n.d.	n.d.	n.d.
Total	**4622**	**1861**	**4620**	**3689**	**3216**	**5393**	**3231**	**3494**

concentrations between 2 ng/L (ClBTR, XTR and Et-*p*-TSA in river samples) and 2,994 ng/L (BTR in influent sewage). Concentrations found in tertiary sewage from treatment based on an ultra-filtration membrane seem to indicate that these treatments could be more effective at removing these contaminants than other tertiary treatments.
N-methyl-*p*-toluenesulfonamide was determined for the first time in influent and secondary and tertiary effluent sewage at concentrations between 2.0 ng/L (tertiary effluent sewage) and 8.6 ng/L (influent sewage).

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3.2.2. A quick, easy, cheap, effective, rugged and safe extraction method followed by liquid chromatography-(Orbitrap) high resolution mass spectrometry to determine benzotriazole, benzothiazole and benzenesulfonamide derivates in sewage sludge

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A QUICK, EASY, CHEAP, EFFECTIVE, RUGGED AND SAFE EXTRACTION METHOD FOLLOWED BY LIQUID CHROMATOGRAPHY-(ORBITRAP) HIGH RESOLUTION MASS SPECTROMETRY TO DETERMINE BENZOTRIAZOLE, BENZOTHIAZOLE AND BENZENESULFONAMIDE DERIVATES IN SEWAGE SLUDGE

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Abstract

A Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method followed by liquid chromatography-(Orbitrap) high resolution mass spectrometry was developed for the simultaneous determination of five benzotriazole, four benzothiazole and five benzenesulfonamide derivates in sewage sludge. While the method was being developed, several buffers and dispersive solid-phase extraction clean-up (dSPE) sorbents were tested. Citrate buffer and Z-sep$^+$ (zirconium-based sorbent) were the most effective extraction buffer and dSPE clean-up material. The absolute recoveries were higher than 80% for all compounds (100 ng/g (d.w.)) and the matrix effect was less than -20% for most compounds. The limits of detection were between 0.5 and 10 ng/g (d.w.) and the limits of quantification (LOQ) were between 1 and 25 ng/g (d.w.). Repeatability and reproducibility were lower than 15% (%RSD, n=5). Several sludge samples from five sewage treatment plants in Catalonia were analyzed and the most abundant compounds were 2-hydroxybenzothiazole (<LOQ-181.2 ng/g (d.w.)) and 4-methyl-1-H-benzotriazole (<LOQ-82.3 ng/g (d.w.)). Benzenesulfonamide (n.d.-70.2 ng/g (d.w.)) and toluenesulfonamide (n.d.-83.9 ng/g (d.w.)) were also determined.

Keywords: *Benzotriazoles; Benzothiazoles; Benzenesulfonamides; QuEChERS; Liquid chromatography-high resolution mass spectrometry; Sewage sludge.*

1 Introduction

Sewage sludge is the solid residue resulting from sewage treatment in sewage treatment plants (STPs) and it is often reused, particularly for agricultural applications, one of the most sustainable options. Sewage comes from such discharging sources as households, industry and hospitals, and sludge contains organic contaminants or their metabolites and transformation products. Therefore, this option for managing sewage sludge may cause the intrusion of organic contaminants into agricultural land, resulting in an ecological and/or health risk [1].

Benzotriazole (BTRs), benzothiazole (BTs) and benzenesulfonamide (BSAs) derivates are well-known aquatic contaminants released from industries and households in, for example, metal corrosion inhibitors or plasticizers. Their presence in sewage has been thoroughly investigated [2-11] but the information about their occurrence in sewage sludge is limited to a few papers [5,12-15] on BTRs and BTs. No information about BSAs has been reported. Some of these contaminants have been classified as toxic to aquatic organisms and bacteria with long-term adverse effects [3,16,17] and they are resistant to biodegradation [18].

Nowadays, laboratories monitor concentrations of several chemical pollutants in a variety of environmental samples. Nevertheless, depending on the analytical method used this work can be tedious so several analytical methods have focused on reducing time and costs. One of these methods is QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), an extraction technique based on solid-liquid extraction that revolutionized the determination of pesticide residues in food matrices [19]. Some authors have suggested that it can be used in various solid matrices [20-25].

The extraction techniques that have most commonly been used with sewage sludge samples are pressurized liquid extraction (PLE), microwave assisted extraction (MAE) and ultrasound assisted solvent extraction (USAE) [1,26]. These techniques provide good extraction efficiencies for several compounds but most of them require sophisticated equipment, take a relatively long time or consume considerable amounts of solvent. Moreover, a solid-phase extraction (SPE) is usually performed after solvent extraction to reduce matrix interferences and improve limits of detection (LODs).

The QuEChERS method has recently been applied to determine a wide range of pharmaceutical compounds [21] in sewage sludge for the first time with very promising results. It has also been used to extract polycyclic aromatic hydrocarbons [27] and chlorinated compounds [23] from soil samples. Nonetheless, it has not yet been applied to other compound families such as BTRs, BTs and BSAs. QuEChERS methods use a single step buffered acetonitrile extraction and simultaneously salt out

water from the aqueous sample using anhydrous magnesium sulphate to induce liquid-liquid partitioning. Subsequently, a clean-up step using a dispersive solid-phase extraction (dSPE) is often conducted to reduce matrix interferences [20]. The most frequent analytical techniques used to determine BTRs, BTs and BSAs are LC [2,3,28] and GC [7,29,30] coupled to MS or MS/MS and HRMS [18,30,31] by Orbitrap based mass analyzers or hybrid Q-TOF analyzers. This study, then, focuses on developing a method that uses QuEChERS extraction (including the study of various dSPE sorbents to reduce the matrix effect in sludge samples) followed by LC-(Orbitrap) HRMS to determine five BTRs, four BTs and five BSAs in sewage sludge. Little is known about the occurrence of these compounds in this kind of sample and this study will extend our knowledge by analyzing several sludge samples from different STPs.

2 Experimental

2.1 Reagents and standards

The chemical standards of five benzotriazole derivates, four benzothiazole derivates and five benzenesulfonamide derivates were purchased from Sigma-Aldrich (St. Louis, USA). They were, respectively 1-H-benzotriazole (BTR), 4-methyl-1-H-benzotriazole (4TTR), 5-methyl-1-H-benzotriazole (5TTR), 5,6-dimethyl-1H-benzotriazole (XTR) and 5-chloro-1-H-benzotriazole (ClBTR); benzothiazole (BT), 2-aminobenzothiazole (NH_2BT), 2-hydroxybenzothiazole (OHBT) and 2 - (methylthio) benzothiazole (MeSBT); and benzenesulfonamide (BSA), *orto*-toluenesulfonamide (*o*-TSA), *para*-toluenesulfonamide (*p*-TSA), *N*-methyl-*para*-toluenesulfonamide (Me-*p*-TSA) and *N*-ethyl-*para*-toluenesulfonamide (Et-*p*-TSA). Stock solutions of individual standards at 1,000 mg/L were prepared in methanol and stored at -20 ºC. A mixed working solution of 10 mg/L was prepared weekly in methanol. The chemical structures of the compounds studied are shown in Figure S1. 2-chlorobenzothiazole (ClBT) and 4-bromobenzenesulfonamide (BrBSA) were tested as internal standards (IS) and were from Sigma-Aldrich. Stock solutions of each IS at 1,000 mg/L and a mixed working IS solution of 100 mg/L were prepared in methanol and stored at -20 ºC.
Ultrapure water was obtained using an ultrapure water purification system from Veolia Waters (Sant Cugat del Vallés, Spain). Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade and supplied by Prolabo (VWR, Llinars del Vallès, Spain). Acetic acid, ammonium acetate and ammonium hydroxide (LC-MS grade) were purchased from Sigma-Aldrich and nitrogen gas was provided by Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

The sewage sludge samples were collected from five STPs in Catalonia (Spain) located in Tarragona (STP1),

Reus (STP2), Castell-Platja d'Aro (STP3), Blanes (STP4), and Palamós (STP5). Two sewage sludge samples were collected from each STP in different months. These STPs receive urban sewage and industrial discharges from a population of between 100,000 and 200,000 inhabitants and they use activated sludge for biological treatment. They all use anaerobic digestion, except for STP3 which uses aerobic digestion. STPs 3, 4 and 5 are equipped with tertiary sewage treatments. Sewage sludge was dehydrated by centrifugation, in most of cases, or by press filters. The sludge samples collected were frozen before being lyophilized and were then crushed in a mortar and pestle and sieved (125 μm) to obtain particles with the same diameter.

Spiked samples for purposes of optimization were prepared by adding the stock mixture of standards in acetone (the required volume to wet and cover the sludge). The solvent was slowly evaporated at room temperature inside an extractor hood and the sample was frequently homogenized during the two days before extraction to ensure good interaction between the compounds and the matrix.

2.3 LC-(Orbitrap)HRMS analysis

An Accela 1250 UHPLC chromatograph coupled to an Orbitrap/Exactive mass analyzer, both from Thermo Fisher Scientific (Bremen, Germany), were used for LC-HRMS measurements. It was equipped with a quaternary pump (1250 bar) and an Accela autosampler, made up of an automatic injector (refrigerated at 10 ºC) and a column oven (heated at 50 ºC). The electrospray interface was a heated electrospray ionization source (HESI-II). The chromatographic separation was achieved with an Ascentis Express C_{18} column (100 x 2.1 mm, 2.7 μm fused core particle size) from Supelco (Sigma-Aldrich) under gradient elution conditions. The mobile phase was ultrapure water/acetonitrile (98:2) 0.1% CH_3COOH (solvent A) and methanol (Solvent B). It started isocratic at 0.5% B for 5.25 min and then increased to 18% in 3.5 min. After remaining constant for a further 1.25 min, it increased to 35% in 6 min and then up to 95% in another 4 min. It then remained constant for 4 min and returned to initial conditions in 1 min. The flow rate was 800 μL/min and the injection volume was 20 μL. All of the compounds were eluted in less than 18 min.

To optimize HRMS measurements, a mixture of all compounds were infusioned in the source with a syringe pump connected with the column flow conditions. Depending on the compound, either precursor ion $[M+H]^+$ or $[M-H]^-$ were monitored to determine the parameters that lead to best response in each mode, positive or negative, in full-scan at a resolution of 50,000 FWHM over a mass-range of 50-500 Da. Thus, the optimized spray voltage was 4 kV in both ionization modes, the capillary voltage was 37.5 V and -

40 V, the tube lens voltage was 90 V and -90 V and the skimmer voltage was 20 V and -25 V, in the positive and negative mode, respectively. Sheath gas was set to 55 AU and auxiliary gas to 20 AU, the transfer tube was set to 350 ºC and the heater temperature was set to 400 ºC in both ionization modes

Four time windows were used. Two in negative mode (0-2.3 min and 4.8-7.6 min) and two in positive mode (2.3-4.8 min and 7.6-20 min) with two scan events in each time window. The scan events were: one in full scan mode (at 50,000 FWHM with an injection time of 250 ms) and the other in all ion fragmentation mode (at 10,000 FWHM with an injection time of 50 ms) at 25 eV the HCD cell in both negative and positive ionization mode. To follow the guidelines of the European Directive 2002/657/EC [32], at least two product ions for each compound (Table S1) were used for confirmation purposes.

2.4 QuEChERS extraction

One gram of freeze-dried sludge was weighed into a 50 mL centrifuge tube from Scharlab (Sentmenat, Spain), 10 mL of cooled water was added to the tube, and the tube was shaken vigorously for 1 min. Then, 10 mL of ACN was added, followed by an extraction salt packet (Scharlab) for the European Committee for Standardization (CEN) extraction method [33], which contains 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate tribasic dyhidrate. The sample tube was shaken vigorously and stirred for 1 min in a vortex. The tube was further centrifuged at 4,000 rpm for 5 min and a 6 mL aliquot of the supernatant phase (ACN layer) was transferred to a 15 mL centrifuge tube containing 500 mg of Z-sep$^+$ dSPE sorbent for clean-up from Supelco (Sigma-Aldrich). The tube was stirred for 1 min in a vortex and centrifuged at 4,000 rpm for 5 min. A 5 mL aliquot was evaporated under N_2 stream to a final volume of 100 μL (c.a.) and reconstituted to 1 mL with aqueous mobile phase. The extract was filtered with a 0.22 μm PTFE syringe filter and analysed by LC-HRMS.

To optimize the extraction experiments, tests were carried out with the extraction salts used in the Association of Analytical Communities (AOAC) method [34] from Waters (Wexford, Ireland), which contained 6 g of anhydrous magnesium sulphate and 1.5 g of sodium acetate, and lab-made non-buffered original method salts [19] consisting of 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride, both from Sigma-Aldrich. To optimize dSPE experiments, tests were carried out on several dSPE tubes containing 150/900 mg of PSA/$MgSO_4$, 150/150/900 mg of PSA/ C_{18}/$MgSO_4$, 150/15/900 mg of PSA/GCB/$MgSO_4$, 500 mg of Z-sep$^+$ and 500 mg of Florisil from Supelco.

3 Results and discussion

3.1 LC-(Orbitrap)HRMS

The LC separation was adapted from a previous paper [2] in which these compounds were determined in sewage by SPE followed by LC-MS/MS. The first part of the gradient was the same as in the previous study but the second part was smoothed to reduce the matrix effect observed for the compounds eluted last with the method previously reported. However, the two toluene-sulfonamide isomers (*orto-* and *para-*) could not be separated under the chromatographic conditions described. Their separation is complex because their polarity leads to low retention with a C_{18} column and, therefore, to poor selectivity. *Orto-* and *para-*toluenesulfonamide, then, were referred to as toluenesulfonamide, a mixture of both isomers.

For high resolution mass spectrometry with an Orbitrap analyzer, the ionization performance (both positive and negative) in HESI was optimized by infusing a mixture of all compounds in solution under chromatographic flow and mobile phase composition. The best parameters are described in the materials and methods section. Tests were carried out on the probe position (side-to-side (-1 to +1), vertical (C or D) and micrometer (1 to 2)), capillary and transfer tube temperature (from 250 to 450 ºC) and sheath (from 50 to 100 AU) and auxiliary gas (from 0 to 50 AU). The values of these parameters were selected as a compromise between the maximum formation efficiencies of precursor ions of all the compounds. The spray voltage (2 to 5 kV), capillary voltage (±10 to ±100 V), tube lens voltage (±50 to ±200 V) and skimmer voltage (±5 to ±50 V) were selected taking into account all the compounds eluted within the same time window and ionization mode. Moreover, for confirmatory purposes collision energies (5 to 70 eV) in HCD were optimized to obtain at least two intense product ions for each compound in all ion fragmentation spectra. The chemical structure of product ions was assigned taking into account the exact mass measured. They are listed in Table S1.

3.2 Mass spectral characterization

All BTR compounds ionize in positive mode as $[M+H]^+$. In all ion fragmentation spectra obtained for these compounds, nitrogen or hydrogen cyanide molecules are lost. Likewise, BT compounds also ionize in positive mode as $[M+H]^+$, except 2-hydroxybenzothiazole which ionizes in both positive and negative mode as $[M+H]^+$ and $[M-H]^-$ with similar intensities. The positive ionization mode was selected for all of the BTs because 2-hydroxybenzothiazole elutes between 5-chloro-1-H-benzotriazole and benzothiazole and there is no time for the windows to change. The group at position two of the heterocyclic ring is linked to the

ionization efficiency of these compounds which is much higher for 2-hydroxybenzothiazole and 2-aminobenzothiazole than for benzothiazole and 2-(methylthio)benzothiazole. Moreover, when these compounds fragment, they lose the substituent group mentioned above plus a carbon atom. An isobaric interference of benzothiazole, due to mobile phase composition, in its exact mass is recorded in the background spectrum resulting in a lower signal/noise ratio than the other compounds. The background signal for this mass has also been observed in other studies in which different mass analyzers were used (e.g. triple quadrupole or time of flight) [2].

The ionization of BSAs depends on whether or not there is an *N*-substituted group. Thus, benzenesulfonamide and toluene-sufonamide ionize in negative mode as $[M-H]^-$ and *N*-ethyl and *N*-methyl-*p*-toluenesulfonamide ionize in positive mode as $[M+H]^+$. For these compounds, the loss of the benzene and sulphur dioxide molecules are the most common fragments observed.

3.3 QuEChERS extraction

Normally a QuEChERS method involves two extraction steps, the first of which, a salting-out liquid-liquid extraction, extracts the analytes of interest from the matrix while the second, a matrix dispersion extraction, carries out a sample clean-up using a dSPE tube.

3.3.1 Buffer selection

The salting-out liquid-liquid extraction is carried out using an organic solvent, usually acetronitrile. A high content of magnesium sulphate in combination with other salts, generally sodium chloride, is also added to increase the solvent partitioning. As well as these salts, a buffer salt may be added (e.g. an acetate buffer for the AOAC 2007.01 official method [34] or a citrate buffer for the CEN 15662 method [33]) to improve the extraction of analytes and to prevent them from degrading under certain pH conditions. The original (non-buffered) QuEChERS method was introduced by Anastassiades *et al.* [19] and used acetonitrile. As has already been mentioned, most published methods using this extraction approach use ACN, although ethyl acetate and dichloromethane have also been used for extracting some compounds from soil [20]. In the present study, acetonitrile was chosen as the extraction solvent.

The QuEChERS method is usually applied to samples with a high water content (for example, fruit and vegetables) but it can be applied to dried samples if the sample weight is reduced and water is added up to the sample weight proposed by the official method [33,34]. One gram of freeze dried sludge was weighed into a 50 mL centrifuge tube and 10 mL of ultrapure water was added prior to the extraction procedure.

Three different extraction salt mixtures were tested using the official

AOAC and CEN methods and the original non-buffered method. One gram of sludge spiked at 200 ng/g (d.w.) was extracted following the procedure described in Section 2.4 to calculate the recoveries obtained with each extraction salt mixture. These recoveries were calculated by comparing the concentration determined with an external standard calibration in spiked sludge and non-spiked samples that were spiked after the extraction procedure. These recoveries were absolute recoveries because they do not take into account the differences caused by the matrix effect, only the extraction yield. The absolute recoveries obtained with the three extraction procedures are shown in Figure 1.

The recoveries obtained for all compounds with the three extraction salts were very similar and they were all close to 90%, except for ClBTR with the non-buffered method which had a recovery of 80%. However, results were slightly better with the citrate buffer (CEN), especially for BTRs. The matrix effect observed using these three extraction salts was also compared. The results were very similar for the three methods tested but varied considerably for each ionization mode of the molecules. BSA and TSA, which ionize in negative mode, had the lowest matrix effect (around -15% for both compounds). The other compounds, which ionize in positive mode, showed higher matrix effect values (-36% for Me-*p*-TSA to -70% for ClBTR) using the citrate buffer.

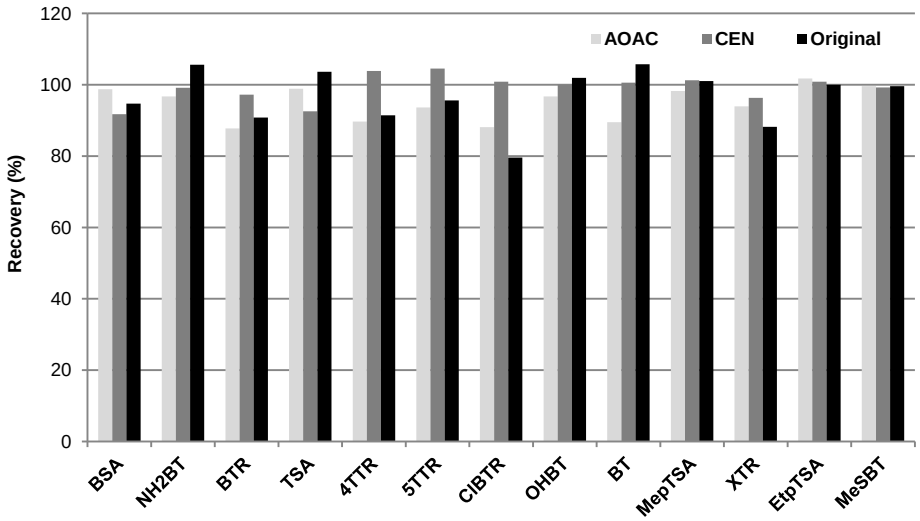

Fig. 1. Absolute recoveries (%) obtained with three different extraction methods based on QuEChERS for spiked sludge at 200 ng/g (d.w.).

Therefore, the citrate buffer was selected for subsequent optimization steps. Extraction time, centrifugation time and speed were not optimized any further because of the excellent recoveries obtained following the specifications of the official methods.

3.3.2 dSPE optimization

One of the major drawbacks in the analysis of sludge samples is the high matrix effect observed in these samples [35]. For this reason, it is often necessary to develop a sample clean-up step before the injection to reduce the matrix constituents. With this in mind, QuEChERS extraction is usually combined with a dSPE so that some matrix constituents can be easily and quickly removed.

dSPE clean-up of an aliquot from the organic layer is conducted using different bulk sorbents, mainly in combination with anhydrous $MgSO_4$. To date, the most common dSPE sorbents used in QuEChERS have been primary-secondary amine (PSA) for the removal of acids, polar pigments and sugars, graphitized carbon black (GCB) for the removal of colour pigments such as chlorophyll, and C_{18} for the removal of lipid and non-polar components [36]. More recently, the C_{18} material was the only sorbent available for the removal of fats and non-polar compounds from samples until the Z-sep$^+$ dSPE sorbent was commercialized by Supelco. This sorbent is based on the interaction of fats by a Lewis acid-base mechanism with a zirconium (Zr) atom attached to silica solid particle and not on the hydrophobic interactions of C_{18} sorbents (Figure S2). This interaction does not depend on the aqueous content in the sample extract resulting in a more efficient and reproducible dSPE clean-up step [37-39]. In the present study, PSA, PSA/C_{18}, PSA/GCB, Z-sep$^+$ and Florisil were tested as dSPE strategies for reducing the matrix effect in sludge samples.

To optimize the dSPE clean-up, several extracts using the aforementioned LLE of non-spiked sludge samples were combined and spiked at 20 ng/mL (which is 200 ng/g in sludge). Then, 6 mL of this solution was transferred to the various dSPE tubes containing the different sorbents. The dSPE tubes were shaken for 1 min in a vortex and centrifuged at 4,000 rpm for 5 min. After this, a 5 mL aliquot was evaporated under a N_2 stream, reconstituted to 1 mL with aqueous mobile phase and analyzed. In addition, a 5 mL aliquot (without clean-up) was evaporated so that it could be compared with the extracts obtained using dSPE. The non-spiked aliquots processed with the different dSPE sorbents were also analyzed and the response of the blanks subtracted. Table 1 shows the apparent recoveries provided by the various dSPE sorbents. They were calculated by comparing the concentration obtained with an external standard calibration with the expected concentration after blank subtraction. The results obtained denote that no significant differences

were observed between the different dSPE sorbents tested. However, they all improve the apparent recoveries by between 10% (BTR) and 40% (MeSBT). The apparent recoveries obtained were between 40% for ClBTR using the PSA/GCB sorbent and 94% for BSA using Florisil. Nonetheless, the apparent recoveries were higher than 70% for most of the compounds. On the basis of these results, we decided to use a Z-sep$^+$ because it is specifically designed to remove interferences from complex matrices and provides more robust LC-MS analysis [38]. Sample clean-up with Z-sep$^+$ was also tested under both acidic and neutral extracts because the addition of 1% formic acid can improve the recovery for some compounds, as is reported in the supplier's information. However, no differences were observed between the two pHs so the addition of formic acid to the sample extract was discarded.

The matrix effect was evaluated by comparing the concentration determined in a sludge extract (n=3), spiked after LLE extraction and dSPE at 50 ng/mL (corresponding to 100 ng/g (d.w.)), with the theoretical concentration expected using an external standard calibration. A non-spiked sludge sample was analyzed so that the response caused by the analytes present in the sample could be subtracted. Thus, the matrix effect ranged from -5% (BSA and TSA) to -43% (XTR) and it is shown for each compound in Table 2. The lower values correspond to analytes ionized in negative mode. This may be due to the greater specificity of the negative ionization mechanism, which results in less ion suppression [40-42]. Nevertheless, for most of the compounds the matrix effect was less than -20%, except for some BTRs which had undergone a little more ion suppression.

Table 1. Apparent recoveries (%) obtained for a spiked sewage sludge at 200 ng/g (d.w.) with and without dSPE clean-up materials tested. For conditions, see text.

Compound	Without	PSA	PSA/C_{18}	PSA/GCB	Z-Sep$^+$	Florisil
BSA	77	92	88	82	85	94
NH_2BT	-	-	-	-	-	-
BTR	57	65	66	64	62	61
TSA	75	90	88	83	83	84
4TTR	41	62	66	57	58	56
5TTR	48	64	67	61	63	65
ClBTR	31	44	55	40	48	45
OHBT	51	70	85	65	79	63
BT	54	71	81	69	77	67
Me-*p*-TSA	66	79	79	77	75	66
XTR	25	45	50	45	46	48
Et-*p*-TSA	50	82	85	79	65	74
MeSBT	31	63	76	58	71	70

%RSD(n=3)<15%

3.3.3 Quantification method

As the method is to be accurate and robust, the most suitable quantification method needs to be assessed and the differences in matrix effect and/or extraction efficiency in different samples need to be minimized. In the present study, two different strategies were tested for quantifying these compounds. One was the use of an internal standard calibration and two compounds were selected as candidates for this purpose. The use of isotopic-labelled compounds was discounted because the commercially available compounds (d_4-BTR, $^{13}C_6$-BSA and *p*-^{15}N-TSA) eluted at the beginning of the chromatogram. Thus, two halogenated compounds (ClBT and BrBSA) from the same family of target compounds which were not present in the samples and not used in industry and/or households were tested for this purpose but they were found not to be suitable for two reasons. ClBT has a very low ionization efficiency and it is only detectable at high concentrations (>500 ng/mL). This concentration is quite different from the expected concentration of the target compounds, so it was discounted. BrBSA shows good ionization in negative mode but its retention time is very near to that of 4TTR which ionizes in positive mode. The separation of the first-eluted compounds and TTR isomers is critical and the gradient slope cannot be changed to increase the resolution between BrBSA and 4TTR. This means that it is impossible to have different time windows between these compounds. Therefore, the use of BrBSA was also discounted.

Another option is to use a matrix-matched calibration. This option is often chosen to analyze complex matrices such as sewage sludge [35,43] so we decided to use it in the present study. This quantification method is explained in the section Method validation but the method was not validated for NH_2BT because of its singular chromatographic behavior (see below).

3.3.4 The case of NH_2BT

NH_2BT was a special case. The absolute recovery obtained for this compound was similar to the others (~90%) but the matrix effect observed was higher than -99%. On the basis of the results obtained for the other compounds of the same family or the compounds eluted near it, this high value was not expected. Nevertheless, when we looked beyond the expected retention time we observed another broad peak with the same exact mass. This peak was carefully examined and the product ions were observed to be NH_2BT. In addition, the sample extract was spiked at different concentrations of NH_2BT and the area of the peak changed according to the concentration added. Therefore, this unexpected peak was assigned to NH_2BT, which interacts with matrix components and increases their retention factor. NH_2BT is the only compound in cationic form in the

Table 1. Apparent recoveries (%) obtained for a spiked sewage sludge sample at 200 ng/g (d.w.) with and without dSPE clean-up materials tested. For conditions, see text.

Compound	Without	PSA	PSA/C_{18}	PSA/GCB	Z-Sep$^+$	Florisil
BSA	77	92	88	82	85	94
NH_2BT	-	-	-	-	-	-
BTR	57	65	66	64	62	61
TSA	75	90	88	83	83	84
4TTR	41	62	66	57	58	56
5TTR	48	64	67	61	63	65
ClBTR	31	44	55	40	48	45
OHBT	51	70	85	65	79	63
BT	54	71	81	69	77	67
Me-*p*-TSA	66	79	79	77	75	66
XTR	25	45	50	45	46	48
Et-*p*-TSA	50	82	85	79	65	74
MeSBT	31	63	76	58	71	70

%RSD(n=3)<15%

acidic composition of the mobile phase and this is expected to be the reason why it interacts with the matrix components. Sewage sludge is a kind of matrix which contains a high concentration of organic matter, and characteristically has a considerable number of carboxylic acids in its structure. Therefore, these anionic groups could interact with NH_2BT via electrostatic bonds.

Several studies were carried out to break this interaction because, as can be observed in the results presented in this study, the dSPE sorbents tested do not solve the problem. In the first instance, the pH values of the aqueous mobile phase became less acidic when ammonium acetate buffered solutions were used (pH 4 and pH 5.5). At pH 5.5 the interaction was completely broken but the ionization of the compounds that ionize in positive mode was considerably reduced and some compounds were not ionized as ClBTR. At pH 4, the interaction was not fully eliminated and the ionization was also reduced. Thus, we decided not to make any changes in the mobile phase pH because the other compounds under study were negatively affected. The behavior of this compound under different mobile phase conditions is shown in Figure 2.

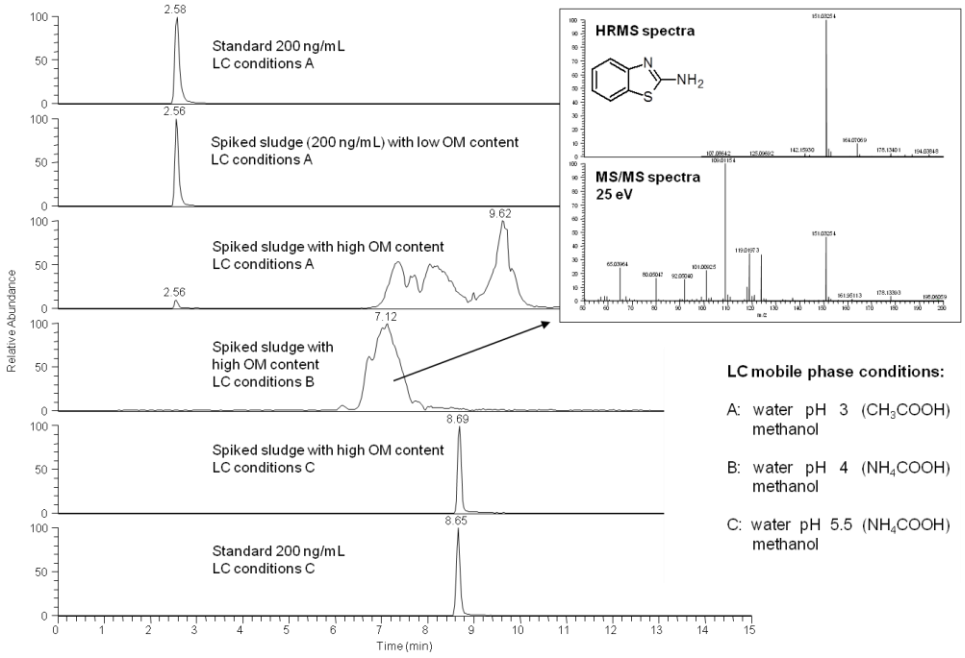

Fig. 2. Behavior of 2-aminobenzothiazole with changes on the mobile phase pH and in sludge samples with different organic matter (OM) content.

Because the matrix components and NH_2BT interact in sample extracts, changes in the pH and organic composition of this extract after the evaporation step were tested. So, acidic (pH 3), neutral (pH 7) or alkaline (pH 9) solutions were used to reconstitute the extracted residue before UHPLC-HRMS analysis. As occurred with the pH of the mobile phase, the solution at pH 9 broke the interaction but not completely. This led to broader peaks for the first compounds eluted due to the considerable difference in pH between the elution plug and mobile phase composition. No improvements were observed when the organic composition was changed.

In addition, sludge samples from different STPs containing different organic matter were tested to determine whether this was a one-off problem or not. The problem was observed for the other matrices tested, except for a sludge sample which contained less organic matter. Hence, we expected this problem to appear in most sludge samples and we consider that the proposed method is not suitable for determining NH_2BT. However, it can be determined by itself with the proposed QuEChERS extraction if the pH of the mobile phase composition in LC is changed to pH 5.5 or higher.

3.4 Method validation

In order to validate the method developed, we calculated the linear range, the limits of quantification and detection, the absolute recoveries for the whole extraction method and the repeatability (intra-day) and reproducibility (day-to-day) for a non-spiked sample and two spiked concentration levels. All of these parameters are shown in Table 2. The linear range was evaluated by constructing a matrix-matched calibration curve with sludge samples spiked at different concentrations (10 calibration points) from 0.5 to 500 ng/g (d.w.) before they were extracted by the QuEChERS method developed. This calibration curve was used to quantify the samples and r^2 was higher than 0.998 for all compounds. A non-spiked sludge sample was analyzed so that the signal of the analytes present in the sample used for calibration could be subtracted (BTR, 4TTR, 5TTR and OHBT). The LOQs were defined as the lowest point of the calibration curve and the LODs corresponded to a signal/noise ratio equal to 3 for the compounds which were not present in the aforementioned sample. The LODs of the compounds that were present in the sample were estimated from their recoveries and the MS response. Thus, the LOQs were between 1 ng/g (d.w.) and 25 ng/g (d.w.)) and the LODs were between 0.5 ng/g (d.w.) and 10 ng/g (d.w.) depending on the compound.

Repeatability (intra-day, n=5) and reproducibility (day-to-day, n=5), expressed as %Relative Standard Deviation (%RSD), were calculated using sludge samples spiked at two concentrations (50 and 250 ng/g

Table 2. Retention time (t_R), limit of detection (LOD) and quantification (LOQ), absolute recovery, matrix effect, repeatability and reproducibility (%RSD, n=5) of the developed method for the studied compounds.

Compound	t_R	LOD	LOQ	Recovery[a]	ME	Repeatability (%RSD, n=5)			Reproducibility (%RSD, n=5)		
	(min)	(ng/g (d.w.))	(ng/g (d.w.))	(%)	(%)	non-spiked	50 ng/g	250 ng/g	non-spiked	50 ng/g	250 ng/g
BSA	1.7	10	25	90	-5	1	3	4	6	4	3
BTR	3.1	0.5	1	85	-27	1	5	4	9	7	3
TSA	5.4	5	10	87	-5	7	9	4	7	4	5
4TTR	8.5	0.5	1	85	-31	4	8	4	3	3	4
5TTR	8.8	0.5	1	86	-26	1	5	3	8	2	3
ClBTR	9.4	0.5	1	80	-40	6	4	2	5	5	4
OHBT	9.8	0.5	1	90	-12	1	11	5	12	7	6
BT	10.2	10	25	87	-12	n.d.	4	4	n.d.	6	3
Me-*p*-TSA	10.5	10	25	88	-15	n.d.	3	5	n.d.	15	8
XTR	12.0	5	10	81	-43	n.d.	10	2	n.d.	5	4
Et-*p*-TSA	13.5	5	10	81	-20	n.d.	4	3	n.d.	2	3
MeSBT	17.9	10	25	89	-21	n.d.	6	5	n.d.	4	5

[a] sludge spiked at 100 n/g (d.w.)
n.d. is not detected

(d.w.)) and a field (non-spiked) sample. Repeatability was below 11% and reproducibility below 15% for all compounds.

Absolute recoveries were determined by comparing the concentrations determined for sludge samples spiked at 100 ng/g (d.w.) (n=5) with a sludge extract from the same sample spiked at 50 ng/mL (to take the dilution factor of the extraction procedure into account) after the extraction process and quantified using the matrix-matched calibration. The recoveries obtained were higher than 80% for all compounds which shows that the method developed can be used to analyse sewage sludge samples.

To date, few papers have been published on the determination of BTRs and BTs in sewage sludge. To the best of our knowledge, the determination of BSAs has not previously been reported. Liu *et al.* [5,10] used PLE/GC-MS/MS and Zhang *et al.* [14] solid-liquid extraction followed by GC-MS and LC-MS/MS to report LODs and LOQs for BTRs between 0.7 and 4.1 ng/g (d.w.) and between 2.2 and 13.8 ng/g (d.w.), respectively. Wick *et al.* [13] used PLE/LC-MS/MS to determine some BTs and obtained LOQs of 100 ng/g (d.w.) for BT and OHBT and 25 ng/g (d.w.) for MeSBT. Moreover, Asimakopoulos *et al.* [12] used USAE followed by UHPLC-MS/MS to simultaneously determine BTRs and BTs and LODs were between 0.04 ng/g (d.w.) for MeSBT and 12.5 ng/g (d.w.) for BT. We obtained similar values using the QuEChERS method. The recoveries obtained in the present paper are as good as or better than those reported in the aforementioned papers using other extraction techniques. Nonetheless, QuEChERS extraction is easier, faster and cheaper than other extraction techniques previously reported for the analysis of sludge samples.

4 Application to sewage sludge samples

The method developed was used to determine five BTRs, three BTs and four BSAs in several sludge samples collected from five STPs. The presence of the compounds detected was confirmed with the exact mass of parent and product ions (Table S1), in accordance with the guidelines of European Directive 2002/657/EC [32]. An HRMS chromatogram of a sewage sludge sample is presented in Figure 3. It shows the compounds found in one analyzed sample from Blanes STP and the corresponding mass error of the precursor ion of each compound is also indicated. These errors are between 1.2 and 4.3 ppm which indicates high confidence in the results obtained when a mass extract window of 5 ppm is used. In this sample, NH_2BT appears in the chromatogram because it is a sample that contains little organic matter but, as explained above, this compound was not quantified.

The concentrations found in the samples analyzed are shown in Table 3. All of the compounds studied were determined, except BT, which was

Table 3. Concentration found (ng/g (d.w.)) of BTRs, BTs and BSAs in sludge samples from investigated STPs.

Compound	STP 1		STP 2		STP 3		STP 4		STP 5	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
BSA	31.4	<LOQ	28.9	38.9	n.d.	75.0	<LOQ	<LOQ	n.d.	70.2
NH_2BT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.*	n.q.*	n.d.	n.d.
BTR	24.4	24.2	27.1	27.0	<LOQ	<LOQ	1.5	<LOQ	11.3	18.6
TSA	<LOQ	<LOQ	18.4	<LOQ	n.d.	n.d.	83.9	71.9	<LOQ	n.d.
4TTR	69.9	82.3	59.5	41.8	4.3	4.4	9.8	3.4	62.6	<LOQ
5TTR	30.4	28.3	20.2	7.4	<LOQ	<LOQ	<LOQ	<LOQ	16.2	7.4
ClBTR	2.3	<LOQ	<LOQ	<LOQ	n.d.	n.d.	<LOQ	<LOQ	<LOQ	<LOQ
OHBT	173.4	181.2	132.4	75.5	<LOQ	<LOQ	<LOQ	<LOQ	129.7	93.3
BT	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.
Me-*p*-TSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
XTR	<LOQ	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ
Et-*p*-TSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MeSBT	25.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	39.8	n.d.

n.d. is not detected
n.q. is not quantified

found below its LOQ, and Me-*p*-TSA and Et-*p*-TSA, which were not detected. The most abundant compound determined was OHBT at concentrations between 75.5 and 181.2 ng/g (d.w.).

Most of STPs used activated sludge with anaerobic digestion, except the STP in Castell-Platja d'Aro (STP3), which uses anaerobic digestion. These compounds occur in the sludge from STP2 and STP3 much less frequently than in the sludge samples from other STPs, as observed in the results obtained. This may be due to the digestion under aerobic conditions in STP3 and the elimination of nitrogen and phosphorous during sewage treatment in STP4, which may cause a major degradation of the compounds under study, or different concentrations in the influent sewage.

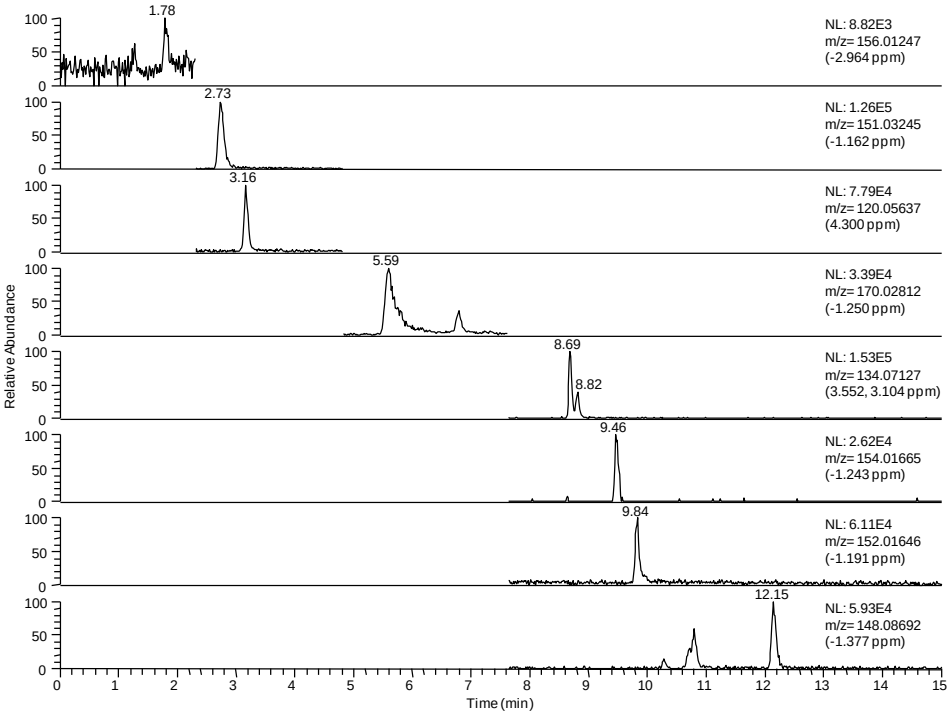

Fig. 3. HRMS chromatogram and mass error in ppm of a sludge sample from STP4 (Blanes).

In addition, sludge samples from STP4 have the lowest concentrations of organic matter and the concentrations found for the target compounds are quite different from those of other STPs that use anaerobic digestion. OHBT was found to be below its LOQ, even though it was the most abundant compound in the other samples, and

TSA was found at higher concentrations than other samples (between 71.9 and 83.9 ng/g (d.w.)). This compound may be formed as a degradation product of some compounds (Chloramin-T, e.g.) and not only as a parent compound or transformation product of other BSAs [44]. Moreover, NH_2BT was detected but not quantified because the method cannot be validated for this compound. Nonetheless, this indicates that it may be present in other sludge samples. The higher concentration observed for OHBT in the samples may be the result of the degradation or transformation of other BTs to OHBT.

The results obtained are similar to the results reported by other authors in sludge samples. Hence, Zhang *et al.* [14] determined BTR at levels between 17.2 and 198 ng/g (d.w.) and 5TTR between 30 and 104 ng/g (d.w.). Liu *et al.* [10] determined BTR, 5TTR, ClBTR and XTR at maximum concentrations of 219, 98, 114 and 161 ng/g (d.w.), respectively. Wick *et al.* [13] found BT, MeSBT and OHBT at 265, 157 and 307 ng/g (d.w.), and Asimakopoulos *et al.* [12] found BTR, TTR, BT, MeSBT and OHBT at 84, 116, 174, 61 and 74 ng/g (d.w.). The results reported in these two papers for BT and MeSBT are quite different to ours (BT and MeSBT were frequently detected below their LOQ). To our knowledge, the present paper is the first to determine BSA and its derivates in sewage sludge. BSA and TSA were determined and Me-*p*-TSA and Et-*p*-TSA were not detected in the samples analyzed, which suggests that these compounds may be degraded during sewage treatments to *p*-TSA or BSA.

5 Conclusions

A QuEChERS based extraction method followed by LC-HRMS to determine simultaneously five benzotriazole, four benzothiazole and four benzenesulfonamide derivates has been developed and validated.

The recoveries of whole method were higher than 80% for all compounds (100 ng/g (d.w.)). Several d-SPE clean-up were tested to reduce the matrix effect and Z-sep$^+$ sorbent were selected as the best option, obtaining ion suppression values less than -20% for most compounds.

The repeatability (intra-day) and reproducibility (day-to-day) of the method was less than 11% and 12% (%RSD, n=5), respectively.

Several compounds were determined in analyzed samples being OHBT the most abundant compound (75.5 and 181.2 ng/g (d.w.)). The occurrence of benzenesulfonamide derivates in sewage sludge was investigated for the first time and BSA and TSA were determined at levels between <LOQ-70.2 ng/g (d.w.) and <LOQ-83.9 ng/g (d.w.), respectively.

Acknowledgments

The authors wish to thank the personnel of the sewage treatment plants for their cooperation in all aspects of this study.

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Supplementary Data

Benzotriazole **Benzothiazole** **Benzenesulfonamide**

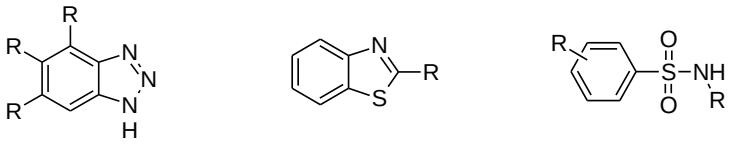

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Fig. S1. Chemical structure of benzotriazole, benzothiazole and benzenesulfonamide derivates.

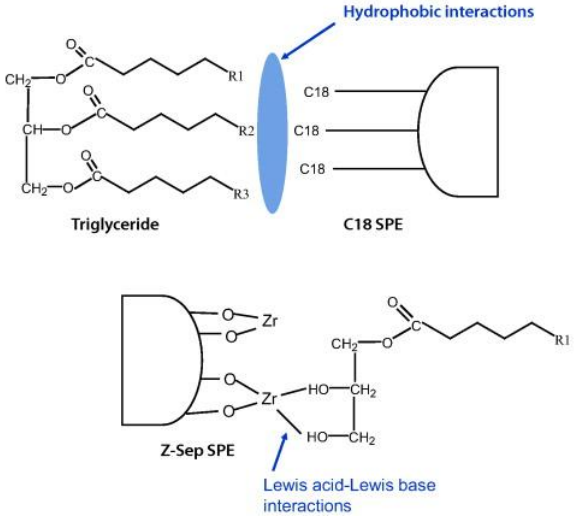

Fig. S2. Z-sep$^+$ and C_{18} dSPE structures and interaction mechanisms (from Supelco).

Table S1. Chemical formula, exact mass of precursor ions and most intense product ions observed of studied compounds.

Compound	Chemical formula	Precursor ion (m/z)	Product ions (m/z)	
Benzotriazoles				
1H-benzotriazole (BTR)	$C_6H_5N_3$	120.05637 $[M+H]^+$	65.03858 92.04984	$C_5H_5^+$ $C_6H_6N^+$
4-methyl-1H-benzotriazole (4TTR)	$C_7H_7N_3$	134.07127 $[M+H]^+$	79.05423 105.04472 77.03858	$C_6H_7^+$ $C_6H_5N_2^+$ $C_6H_5^+$
5-methyl-1H-benzotriazole (5TTR)	$C_7H_7N_3$	134.07127 $[M+H]^+$	79.05423 105.04472 77.03858	$C_6H_7^+$ $C_6H_5N_2^+$ $C_6H_5^+$
5,6-dimethyl-1H-benzotriazole (XTR)	$C_8H_9N_3$	148.08692 $[M+H]^+$	91.05423 93.06988 105.04472	$C_7H_7^+$ $C_7H_9^+$ $C_6H_5N_2^+$
5-chloro-1H-benzotriazole (ClBTR)	$C_6H_4N_3{}^{35}Cl$ $C_6H_4N_3{}^{37}Cl$	154.01665 $[M+H]^+$ 156.01371 $[M+H]^+$	98.99960 90.03383 100.99665	$C_5H_4{}^{35}Cl^+$ $C_6H_4N^+$ $C_5H_4{}^{37}Cl^+$
Benzothiazoles				
Benzothiazole (BT)	C_7H_5NS	136.02155 $[M+H]^+$	109.01065 65.03858	$C_6H_5S^+$ $C_5H_5^+$
2-aminobenzothiazole (NH_2BT)	$C_7H_6N_2S$	151.03245 $[M+H]^+$	109.01065 65.03858 124.02155	$C_6H_5S^+$ $C_5H_5^+$ $C_6H_5SN^+$
2-hydroxybenzothiazole (OHBT)	C_7H_5NSO	152.01646 $[M+H]^+$	124.02155 92.04984 109.01065	$C_6H_5SN^+$ $C_6H_6N^+$ $C_6H_5S^+$
2-(methylthio)benzothiazole (MeSBT)	$C_8H_7NS_2$	182.00927 $[M+H]^+$	166.98579 109.01065	$C_7H_5S_2N^+$ $C_6H_5S^+$

Table S1. (Cont.).

Compound	Chemical formula	Precursor ion (m/z)	Product ions (m/z)	
Benzenesulfonamides				
Benzenesulfonamide (BSA)	$C_6H_7NSO_2$	156.01247 [M-H]$^-$	92.05057 78.97335	$C_6H_6N^-$ SO_2NH^-
Toluenesulfonamide (TSA)	$C_7H_9NSO_2$	170.02812 [M-H]$^-$	106.06622 78.97335	$C_7H_8N^-$ SO_2NH^-
N-methyl-*p*-toluenesulfonamide (Me-*p*-TSA)	$C_8H_{11}NSO_2$	186.05833 [M+H]$^+$	91.05423 64.96918	$C_7H_7^+$ SO_2H^+
N-ethyl-*p*-toluenesulfonamide (Et-*p*-TSA)	$C_9H_{13}NSO_2$	200.07398 [M+H]$^+$	91.05423 64.96918	$C_7H_7^+$ SO_2H^+

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Dipòsit Legal: T 1619-2015

3.3.3. A pressurised hot water extraction and liquid chromatography-high resolution mass spectrometry method to determine polar benzotriazole, benzotriazole and benzenesulfonamide derivates in sewage sludge

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A PRESSURISED HOT WATER EXTRACTION AND LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY METHOD TO DETERMINE POLAR BENZOTRIAZOLE, BENZOTHIAZOLE AND BENZENESULFONAMIDE DERIVATES IN SEWAGE SLUDGE

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Abstract

Benzothiazole, benzotriazole and benzenesulfonamide derivates are well-known aquatic contaminants, although very few studies have been published about their occurrence in sewage sludge samples. In this paper, a pressurised hot water extraction (PHWE) method has been developed for the simultaneous determination of these families of compounds. The compounds were determined by LC-Orbitrap-HRMS and several clean-up strategies such as in-cell PHWE and solid-phase extraction (SPE) were tested to reduce the high matrix effect that occurs when sludge samples are analysed. Absolute recoveries using the whole method were above 80% and the matrix effect was under -20% for most of the compounds studied. Repeatability and reproducibility were usually under 10% (%RSD, 50 and 250 ng g^{-1} (d.w.), n=5), while LODs and LOQs were between 0.25 and 25 ng g^{-1} (d.w.) and 0.5 and 50 ng g^{-1} (d.w.) respectively. The PHWE/SPE/LC-HRMS method developed was used to analyse several sludge samples collected from five sewage treatment plants (STPs) in Catalonia that use different sewage treatments. The most frequently determined compounds were benzotriazole derivates and the most abundant compound found was 2-hydroxybenzothiazole.

Keywords: *Benzotriazoles; Benzothiazoles; Benzenesulfonamides; Pressurised hot water extraction; Liquid chromatography-Orbitrap-high resolution mass spectrometry; Sewage sludge.*

1 Introduction

The widespread use of benzotriazole (BTRs), benzothiazole (BTs) and benzenesulfonamide (BSAs) derivates in everyday consumer products and industrial applications along with their high water solubility and resistance to biodegradation means that these compounds are considered to be ubiquitous water contaminants [1-3].

These chemicals are used as anticorrosive additives in several anti-icing fluids and as silver protectors in dishwasher detergents (BTRs), as vulcanisation accelerators and biocides in several manufacturing industries (BTs) and as plasticisers and intermediate synthesis products for pesticides, drugs and saccharine (BSAs). Their occurrence in sewage sludge is therefore to be expected as a result of the high concentrations found in sewage. This hypothesis is supported by the few studies that have reported the presence of certain benzotriazole and benzothiazole derivates in sewage sludge [4-10]. To the best of our knowledge, the presence of benzenesulfonamide derivates in sewage sludge has only been reported by us, and in a very recent research paper [10]. The fate of these contaminants is of interest because of their toxic effects.

BTRs, for example, are phytotoxic, mutagenic to bacteria, toxic to microorganisms and have estrogenic effects in fish, while 1-H-benzotriazole is suspected to be a human carcinogen [11]. BTs have been shown to be dermal sensitisers and are also linked to mutagenicity in microorganisms and to carcinogenicity in humans [11]. In the case of BSAs, toxicity studies exist only for *para*-toluenesulfonamide (*p*-TSA), which has been shown to be moderately toxic, but the Organisation for Economic Co-operation and Development (OECD) has recommended additional tests if large amounts of *p*-TSA are used in the future [12].

To find out more about the occurrence of BTRs, BTs and BSAs in sewage sludge, we need to develop reliable and efficient analytical methods. With this in mind, the extraction technique selected is one of the most important factors, and over the last few years pressurised liquid extraction (PLE), microwave assisted extraction (MAE) and ultrasound assisted extraction (USAE) have become the most suitable options for sludge samples [13].

A QuEChERS method for extracting BTRs, BTs and BSAs in sewage sludge samples was also recently proposed [10]. However, all these techniques usually involve the use of organic solvents. As a result, solvent-free extraction methods have become more popular in order to eliminate or reduce organic solvent consumption during the extraction procedure. A sub-technique of PLE that uses hot water instead of organic solvents, known as pressurised hot water extraction (PHWE), has become a powerful technique for extracting a wide range of polar organic pollutants from environmental solid matrices

[14,15]. Under high temperatures and controlled pressure conditions, the polarity of water can be changed to resemble that of alcohol [16], which is one of the most frequently used solvents for extracting a wide variety of organic contaminants from sludge. The great complexity of sludge samples is well known and it is therefore usual to apply one or more clean-up steps after extraction to reduce the matrix effect when they are analysed using LC-MS methods [17].

These clean-up steps are commonly based on the use of balanced polar non-polar polymeric sorbents or mixed-mode sorbents for solid phase extraction procedures, and therefore extracts containing organic solvents are not fully compatible with these options. Thus the water extracts obtained when PHWE is used are better for subsequent SPE procedures.

With regard to chromatographic analysis, liquid chromatography (LC) [18-21] and gas chromatography (GC and GCxGC) [7,22-24] have been used coupled to mass spectrometry analysers to determine the aforementioned compounds in environmental samples. Different mass analysers have also been used, such as triple quadrupole (QqQ) [6], time-of-flight (TOF) [24] and quadrupole time-of-flight (QqTOF) [25] in gas chromatography and QqQ [8,20] and Orbitrap [3,10] in liquid chromatography. Nevertheless, liquid chromatography coupled to high resolution mass spectrometry with a soft ionisation source is the best approach for determining these compounds because of their high polarity and low mass.

The aim of this study is to develop an analytical method based on PHWE followed by liquid chromatography-(Orbitrap)high resolution mass spectrometry (LC-HRMS) to simultaneously determine five BTRs, four BTs and five BSAs in the highly complex matrix that is sewage sludge. Moreover, various clean-up strategies such as in-cell clean-up during or after PHWE, multi-stage SPE clean-up and combination of both are also tested due to the great complexity of sludge samples. The use of hot water as extractant in combination with an efficient clean-up stage can improve the limits of detection and reduce the matrix effect observed in previously published methods for the determination of these compounds in sludge samples.

The method developed will then be applied to analyse several sludge samples from five STPs located in Catalonia (Spain) to expand the knowledge about the occurrence of these compounds in sewage sludge.

2 Materials and methods

2.1 Reagents and standards

The chemical standards of five benzotriazole derivates: 1-H-benzotriazole (BTR), 4-methyl-1-H-benzotriazole (4TTR), 5-methyl-1-H-benzotriazole (5TTR), 5,6-dimethyl-1-H-benzotriazole (XTR) and 5-chloro-1-H-benzotriazole (ClBTR); four benzothiazole derivates:

benzothiazole (BT), 2-aminobenzothiazole (NH_2BT), 2-hydroxibenzothiazole (OHBT) and 2-(methylthio)benzothiazole (MeSBT); and five benzenesulfonamide derivates: benzenesulfonamide (BSA), *orto*-toluenesulfonamide (*o*-TSA), *para*-toluenesulfonamide (*p*-TSA), *N*-methyl-*para*-toluenesulfonamide (Me-*p*-TSA) and *N*-ethyl-*para*-toluenesulfonamide (Et-*p*-TSA), were purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of individual standards at 1,000 mg L^{-1} were prepared in methanol and stored at -20ºC. A mixed working solution of 10 mg L^{-1} was prepared weekly in methanol.

Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallés, Spain). Acetonitrile (ACN) and methanol (MeOH) were HPLC grade and supplied by Prolabo (VWR, Llinars del Vallès, Spain). Acetic acid, formic acid and ammonium hydroxide (LC-MS grade) were purchased from Sigma-Aldrich and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

The sewage sludge samples were collected from five sewage treatment plants (STPs) in Catalonia (Spain) located in Tarragona (STP1), Reus (STP2), Castell-Platja d'Aro (STP3), Blanes (STP4), and Palamós (STP5). Two samples were collected on different months from each STP. These STPs receive urban sewage and industrial discharges from a population of between 100,000 and 200,000 and use activated sludge for biological treatment. The samples were a mix of primary and secondary sewage sludge, which, after collection, were frozen, lyophilised, crushed and sieved (125 μm) to obtain particles of the same diameter.

Spiked samples for optimisation purposes were prepared by adding the stock mixture of standards in acetone (the volume required to wet and cover the sludge). The solvent was slowly evaporated at room temperature inside an extractor hood with frequent homogenisation of the sample throughout the two days prior to extraction to ensure good interaction between the compounds and the matrix.

2.3 LC-Orbitrap-HRMS analysis

An Accela 1250 UHPLC chromatograph coupled to an Orbitrap/Exactive mass analyser, both from Thermo Fisher Scientific (Bremen, Germany), was used for LC-HRMS measurements. It was equipped with a quaternary pump (1250 bar) and an Accela autosampler, comprising an automatic injector (refrigerated at 10ºC) and a column oven (heated at 50ºC). The electrospray interface was a heated electrospray ionisation source (HESI-II). The chromatographic separation was achieved using an Ascentis Express C_{18} fused-core column (100 x 2.1 mm, 2.7 μm) from Supelco (Sigma-Aldrich) under gradient elution conditions. Ultrapure

water/ACN (98:2) 0.1% CH_3COOH (solvent A) and methanol (solvent B) was the mobile phase and started isocratic at 0.5% B for 5.25 min and then increased to 18% in 3.5 min, remaining constant for a further 1.25 min before increasing to 35% in 6 min and up to 95% in 4 min more. It then remained constant for 4 min and returned to initial conditions in 1 min. The flow rate was 800 μL/min and the injection volume was 20 μL. All the compounds were eluted in less than 18 min. The LC-Orbitrap-HRMS method used was the same than the used in a previous paper [10]. For HRMS measurements, four time windows were used: two in negative mode (0-2.3 min and 4.8-7.6 min) and two in positive mode (2.3-4.8 min and 7.6-20 min). Two scan events took place in each time window: one in full scan mode (at 50,000 FWHM with 250 ms of injection time) and one in all-ion fragmentation mode (at 10,000 FWHM with 50 ms of injection time), with the HCD cell at 25 eV in both negative and positive ionisation mode windows. Following the guidelines laid down in European Directive 2002/657/EC [26], at least two product ions for each compound (Table 1) were used for confirmation purposes. Spray voltage was set at 4 kV in both ionisation modes, capillary voltage at 37.5 V and -40 V, tube lens voltage at 90 V and -90 V and skimmer voltage at 20 V and -25 V. Sheath gas was set at 55 AU and auxiliary gas at 20 AU, while the transfer tube was set at 350ºC and heater temperature at 400ºC.

2.4 Extraction procedure

One gram of freeze-dried sludge was mixed with one gram of diatomaceous earth, and a glass fibre filter was placed at the bottom of an 11 mL stainless steel extraction cell. This extraction cell was then filled with 1 g of diatomaceous earth, the aforementioned mixture of sludge and another gram of diatomaceous earth. A glass fibre filter was placed at the top of the cell and it was then compacted and closed before extraction using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA). The extraction was carried out with one cycle using ultrapure water at 80ºC and 1500 psi for 5 min. The preheating time was 5 min, flush volume was 60% of cell volume and purge time was 120 s.

The aqueous extract (~20 mL) was preconcentrated using an Oasis HLB cartridge (150 mg, 6 cc) from Waters (Wexford, Ireland). The cartridge was preconditioned with 5 mL of MeOH followed by 5 mL of ultrapure water prior to loading the sample. Before the elution step, 5 mL of ultrapure water was passed through the sorbent, which was then dried under vacuum. Next the cartridge was connected to the top of a lab-made Florisil (bulk adsorbent from Sigma-Aldrich) cartridge (500 mg, 6 cc), which had previously been conditioned with 5 mL of MeOH. The analytes were then eluted from the Oasis HLB cartridge with 2x3 mL of MeOH and the eluate was passed through the Florisil cartridge.

Table 1. Chemical formula, theoretical and measured (in brackets) exact mass of precursor ions and most intense product ions observed.

Compound	Precursor ion (m/z)	Chemical formula	Product ions (m/z)	Chemical formula
Benzotriazoles				
1H-benzotriazole (BTR)	120.05637 (120.05630)	$C_6H_6N_3^+$	65.03858 92.04984	$C_5H_5^+$ $C_6H_6N^+$
4-methyl-1H-benzotriazole (4TTR)	134.07127 (134.07190)	$C_7H_8N_3^+$	79.05423 105.04472 77.03858	$C_6H_7^+$ $C_6H_5N_2^+$ $C_6H_5^+$
5-methyl-1H-benzotriazole (5TTR)	134.07127 (134.07196)	$C_7H_8N_3^+$	79.05423 105.04472 77.03858	$C_6H_7^+$ $C_6H_5N_2^+$ $C_6H_5^+$
5,6-dimethyl-1H-benzotriazole (XTR)	148.08692 (148.08702)	$C_8H_{10}N_3^+$	91.05423 93.06988 105.04472	$C_7H_7^+$ $C_7H_9^+$ $C_6H_5N_2^+$
5-chloro-1H-benzotriazole (ClBTR)	154.01665 (154.01678)	$C_6H_5N_3{}^{35}Cl^+$	98.99960 90.03383	$C_5H_4{}^{35}Cl^+$ $C_6H_4N^+$
	156.01371 (156.01355)	$C_6H_5N_3{}^{37}Cl^+$	100.99665	$C_5H_4{}^{37}Cl^+$
Benzothiazoles				
Benzothiazole (BT)	136.02155 (136.02168)	$C_7H_6NS^+$	109.01065 65.03858	$C_6H_5S^+$ $C_5H_5^+$
2-aminobenzothiazole (NH_2BT)	151.03245 (151.03244)	$C_7H_7N_2S^+$	109.01065 65.03858 124.02155	$C_6H_5S^+$ $C_5H_5^+$ $C_6H_5SN^+$
2-hydroxybenzothiazole (OHBT)	152.01646 (152.01659)	$C_7H_6NSO^+$	124.02155 92.04984 109.01065	$C_6H_5SN^+$ $C_6H_6N^+$ $C_6H_5S^+$
2-(methylthio)-benzothiazole (MeSBT)	182.00927 (182.00937)	$C_8H_8NS_2^+$	166.98579 109.01065	$C_7H_5S_2N^+$ $C_6H_5S^+$
Benzenesulfonamides				
Benzenesulfonamide (BSA)	156.01247 (156.01198)	$C_6H_6NSO_2^-$	92.05057 78.97335	$C_6H_6N^-$ SO_2NH^-
Toluenesulfonamide (TSA)	170.02812 (170.02765)	$C_7H_8NSO_2^-$	106.06622 78.97335	$C_7H_8N^-$ SO_2NH^-
N-methyl-*p*-toluenesulfonamide (Me-*p*-TSA)	186.05833 (186.05850)	$C_8H_{10}NSO_2^+$	91.05423 64.96918	$C_7H_7^+$ SO_2H^+
N-ethyl-*p*-toluenesulfonamide (Et-*p*-TSA)	200.07398 (200.07417)	$C_9H_{14}NSO_2^+$	91.05423 64.96918	$C_7H_7^+$ SO_2H^+

The eluate was concentrated (100 μL, ca.) under a flow of nitrogen gas and made up to 1 mL with ultrapure water. The extract was filtered (0.22 μm PTFE) and injected into the LC-HRMS system.

3 Results and discussion

3.1 LC-Orbitrap-HRMS

The LC separation was adapted from a previous paper [20] in which these compounds were determined in sewage by LC-MS/MS, and the gradient elution was transferred to the LC system used in this paper. However, the gradient was further optimised to reduce the matrix effect observed when sludge samples are analysed. This optimisation of the gradient elution is explained in Section 3.3 and the experiments carried out in previous sections were done with the gradient detailed in the paper mentioned above [20]. After optimisation, the gradient which resolves more coeluting components and shorter analysis time was the same than in a previous paper [10], in which these compounds were determined in sludge using a QuEChERS extraction. The operational Orbitrap parameters were the same as those the previous paper [10].

To summarise the mass spectral characterisation, all the compounds ionise under positive mode as $[M+H]^+$ except BSA and TSA, which ionise under negative mode as $[M+H]^-$. In all the ion fragmentation spectra obtained for these compounds, losses of the nitrogen (N_2) or hydrogen cyanide molecule (HCN) are common for BTR derivates, losses of the 2-substituted group in the heterocyclic ring with a carbon atom are common for BT derivates, and losses of the benzene or sulphur dioxide molecule (SO_2) are the most common fragments observed for BSA derivates. In addition to this, 2-hydroxybenzothiazole also ionises in negative mode with a similar intensity to its ionisation in positive mode, but elutes nearby to 5-chloro-1-H-benzotriazole without there being time to make two different time windows. Positive ionisation was therefore selected for the OHBT compound.

3.2 PHWE optimisation

Because of its high dielectric constant (ε=80) at 25ºC and atmospheric pressure, water has not been considered an efficient extracting solvent for organic contaminants. However, if the temperature and pressure are increased, the dielectric constant of water decreases to the values of certain alcohols (ε=24-32) [16]. This is the phenomenon that makes PHWE a reliable technique for extracting a wide variety of organic compounds. The most important extraction parameters to optimise in the PHWE method using a pressurised liquid extraction system are extraction temperature and time [27]. In our experiments the pressure was set at 1,500 psi to change the polarity of the water via temperature

only. Different water pHs and extraction cycles were also tested because they may have a significant effect on extraction performance. Based on our previous experience, other less important parameters that are known not to significantly affect the recoveries, such as flush volume and purge time, were kept at the values mentioned in Section 2.4. For these experiments, the aqueous PHWE extracts obtained (15-18 mL) were made up to 25 mL with ultrapure water.

To optimise the extraction temperature and time, an experimental design was used. The variable values ranged from 40ºC to 160ºC for temperature and from 5 to 15 min for time, in intervals of 40ºC and 5 min. The absolute recoveries were calculated comparing the response obtained for each compound from the spiked sludge (2,000 ng g^{-1} (d.w.)) with a sludge sample spiked after PHWE in order to minimise the differences caused by the matrix effect at different temperatures and times. The absolute recovery values obtained were used to plot an estimated response surface graphic through the ranges tested, using Minitab® (v. 16.1.0, Minitab Inc.) software. For example, the surface obtained for 4TTR is shown in Figure 1.

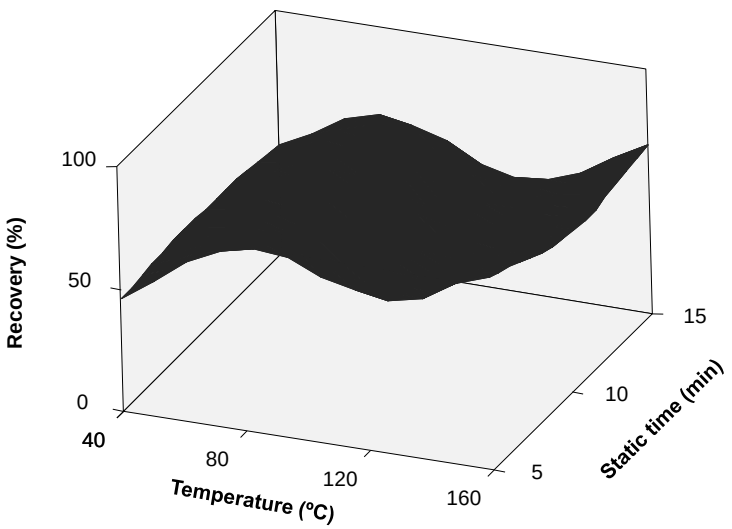

Fig 1. Surface response obtained for 4TTR in PHWE optimisation.

It can be seen that maximum extraction efficiency is obtained at 80ºC and that extraction time does not have a strong impact on extraction performance. For benzothiazole (except MeSBT) and benzenesulfonamide derivates, maximum extraction efficiency was obtained at between 40ºC and 120ºC, and between 80ºC and 160ºC for

benzotriazole derivates, especially ClBTR and XTR. For BTs and BSAs, the recoveries obtained decreased when the temperature was increased by more than 120ºC, probably due to thermal decomposition under aqueous media. MeSBT is the only compound that increases its recovery with temperature, this being 20% at 40ºC and 50% at 160ºC, but temperatures above 80ºC decrease the recoveries for other compounds. On the basis of the results obtained, 80ºC and 5 min were selected as compromise values for all compounds. Under these conditions, the absolute recoveries obtained were close to 90% or above for most compounds except ClBTR (70%), XTR (61%) and MeSBT (26%). The matrix effect was also preliminary evaluated at the different temperatures. Briefly, it was observed that the matrix effect increased two or three times when temperature increased from 40ºC to 160ºC and the colour was also darker at high temperature. Therefore, the extraction conditions selected seem a reasonable option taking into account the recovery yields obtained.

In addition, three different water pHs were tested (3, 7 and 9). These were adjusted using formic acid and ammonium hydroxide because the use of strong acids is not recommended under equipment specifications. The absolute recoveries obtained were very similar for the three pHs tested, but the matrix effect was slightly higher at a pH of 9. Therefore ultrapure water without pH adjustment was selected due to its simplicity. Different extraction cycles were also tested, with additional extractions of the same sample being performed with fresh ultrapure water to increase extraction efficiency. With two cycles, only ClBTR, XTR and MeSBT improve their recovery between 3% and 5%, and with three cycles no improvements were observed in comparison with two cycles. One extraction cycle was therefore selected to save time.

3.3 Clean-up strategies

Due to the matrix effect observed during PLE optimisation, especially for the last eluted compounds, different clean-up strategies were tested to reduce it and achieve better quantification of the target analytes. In-cell clean-up, solid-phase extraction (SPE) clean-up and improvement of the chromatographic separation were therefore proposed, and this section is divided into three sub-sections according to the method stages involved.

To carry out the experiments, 1 g of spiked sludge at 250 ng g^{-1} was extracted by PHWE under the aforementioned conditions. For in-cell clean-up the sludge was mixed with 500 mg of sorbent and placed in the extraction cell. For SPE clean-up, lab-made cartridges were packed using 500 mg of bulk sorbent. The elution of Oasis HLB cartridges was carried out using methanol, as previously explained in Section 2.4. The extract obtained after SPE was concentrated (~100 μL) under an N_2

stream and made up to 1 mL with ultrapure water. The recoveries were calculated using an external standard calibration method to obtain the apparent recoveries which includes the recovery yield and matrix effect contribution [28]. In addition, the apparent recovery obtained without clean-up was used to assess the improvement obtained with each clean-up strategy.

In-cell clean-up

In-cell clean-up requires the use of a sorbent that retains the interfering substances. The sorbent is therefore mixed with the sample and the extraction and clean-up is done in a single step. The sorbents tested were Florisil and C_{18} bulk sorbent, chosen because of the polar characteristics of the compounds and from previous experience. The results are shown in Figure 2. In-cell clean-up does not improve the results obtained without any clean-up strategy because under aqueous conditions Florisil does not retain the interfering substances, so no reduction in the matrix effect was observed, while C_{18} partially retains the target analytes, especially the less polar compounds, reducing the apparent recoveries obtained. Therefore the use of in-cell clean-up was rejected.

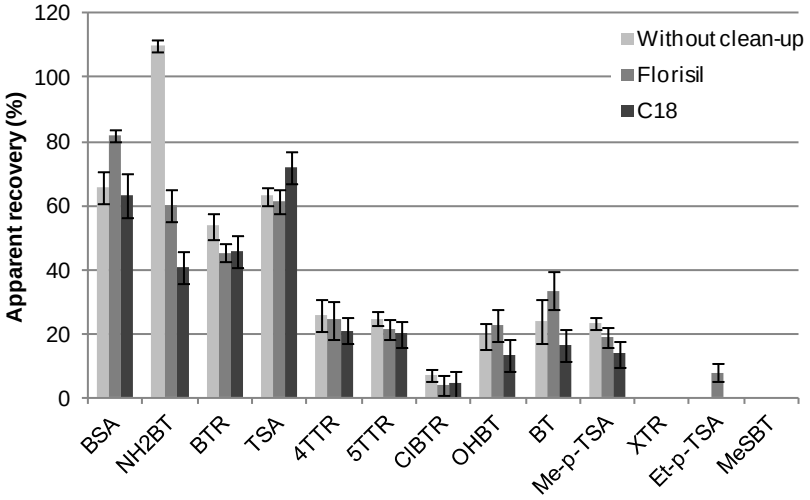

Fig. 2. Apparent recovery (%) for target compounds with Florisil, C_{18} and without in-cell clean-up. For conditions see text.

SPE clean-up

Since the sample extract obtained from PWHE is aqueous, it is simple to perform an SPE procedure using a balanced polar non-polar polymeric sorbent (Oasis HLB), which is the most suitable option due to the polar characteristics of the analytes. These compounds were already determined

in environmental waters in a previous paper [20] using an Oasis HLB sorbent in tandem with a Florisil cartridge to reduce the matrix effect. Therefore this option was tested on the sludge extracts. Together with this procedure as a clean-up agent after Oasis HLB, C_{18} was compared to Florisil in order to find the best option.

The results are shown in Figure 3 and are better than those obtained with in-cell clean-up and improve on the results obtained when no clean-up strategy was adopted. Both Florisil and C_{18} reduce matrix interferences and therefore the apparent recoveries increase. However, Florisil gave much better results than C_{18} for several compounds and two or threefold increases in apparent recoveries were obtained for some compounds. On the basis of these results, tandem SPE consisting of an Oasis HLB cartridge plus a lab-made Florisil cartridge was selected as the best option.

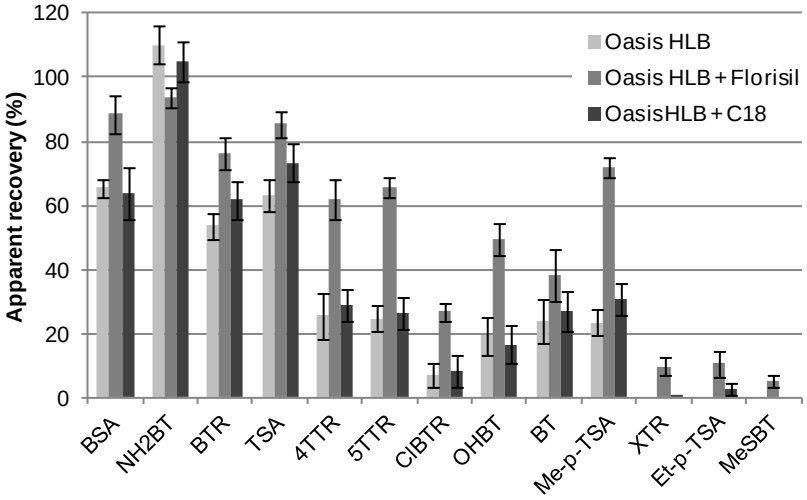

Fig. 3. Apparent recovery (%) for target compounds with Florisil, C_{18} and without tandem SPE clean-up. For conditions see text.

Influence of chromatographic separation

However, the apparent recoveries obtained for the last eluted compounds were very low due to the high matrix effect (up to -90%) in this part of the chromatogram (Figure 4) and/or the low recovery yield for some compounds (MeSBT). Then, modifications of the elution gradient used in previous paper using a SPE/LC-MS/MS method to analyse sewage samples [20] were tested here to reduce matrix effect. The gradient elution slope was smoothed after elution of TTR isomers so as not to modify their separation and to improve the separation of the other compounds from coeluting substances. The best separation was

achieved with a 25 min run time and was the same method as also used for QuEChERS [10]. The improvement obtained can be seen in Figure 4, which shows a chart of the matrix effect observed with the earlier and the optimised chromatographic separations. With optimised separation, the matrix effect for the last eluted compounds was reduced from around -90% to around -20%, with the matrix effect for most of the compounds being less than -20%. With the results obtained after gradient optimisation, the method was validated using a matrix-matched calibration to reduce the differences caused by the matrix effect and/or the method's recovery yield, enabling a more accurate quantification to be obtained. This procedure was also adopted to avoid the use of deuterated surrogates or other internal standard compounds, which are not a reliable option for these compounds, as discussed in previous studies [10,20].

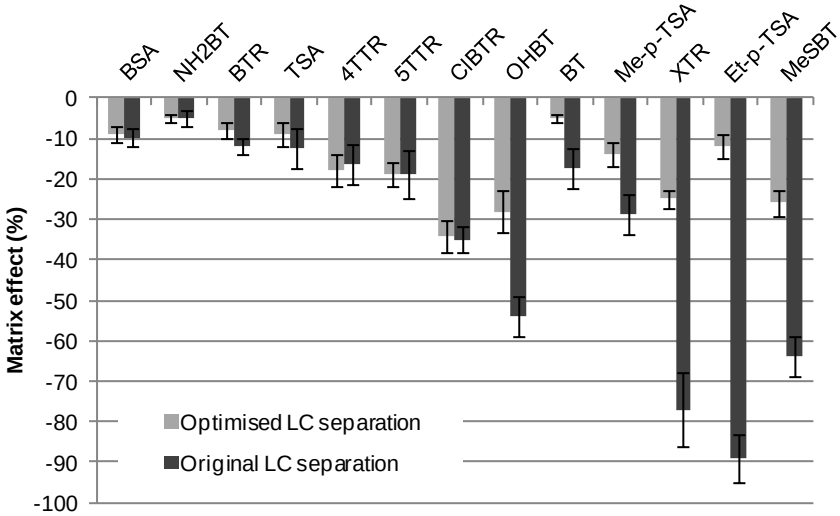

Fig. 4. Matrix effect (%) for target compounds with original and modified chromatographic separation. For conditions see text.

3.4 Method validation

Linear range, method limits of quantification (LOQs) and detection (LODs), repeatability (intra-day), reproducibility (day-to-day) and absolute recoveries for the compounds under study were determined in order to assess the suitability of the method developed for determining these compounds in sewage sludge samples. All these parameters are shown in Table 2. The linear range was evaluated using a matrix-matched calibration curve with sludge samples spiked at different concentrations from 0.25 to 500 ng g^{-1} (d.w.) prior to PHWE

Table 2. Validation values for the PHWE/SPE/LC-Orbitrap-HRMS method.

Compound	t_R (min)	LOD (ng g^{-1} (d.w.))	LOQ (ng g^{-1} (d.w.))	Matrix effect (%)	Absolute Recovery (%, n=5)		Repeatability (%RSD, n=5)		Reproducibility (%RSD, n=5)	
				100 ng g^{-1} (d.w.)	50 ng g^{-1} (d.w.)	250 ng g^{-1} (d.w.)	50 ng g^{-1} (d.w.)	250 ng g^{-1} (d.w.)	50 ng g^{-1} (d.w.)	250 ng g^{-1} (d.w.)
BSA	1.7	5	10	-9	94	102	7	4	10	10
NH_2BT	2.5	1	5	-5	96	101	2	6	6	8
BTR	3.1	0.25	0.50	-8	84	89	5	5	9	5
TSA	5.4	5	10	-9	99	97	7	5	8	7
4TTR	8.5	0.25	0.50	-18	86	81	12	13	12	5
5TTR	8.8	0.25	0.50	-19	83	79	10	9	4	3
ClBTR	9.4	0.25	0.50	-34	44	39	9	8	7	2
OHBT	9.8	0.25	0.50	-28	107	107	7	9	10	4
BT	10.2	5	10	-28	58	54	13	4	8	3
Me-*p*-TSA	10.5	1	5	-14	101	101	7	2	7	5
XTR	12.0	0.25	0.50	-25	45	41	14	14	11	8
Et-*p*-TSA	13.5	1	5	-12	95	101	3	1	3	2
MeSBT	17.9	25	50	-26	25	29	15	13	20	15

extraction. Linear range was from LOQ to 500 ng g^{-1} (d.w.) for each compound with r^2 higher than 0.998 for all compounds in this range. A non-spiked sludge sample was also analysed to subtract any signal of analytes present in the sample (BTR, 4TTR and 5TTR were found at a few ng g^{-1} (d.w.) and OHBT at around 40 ng g^{-1} (d.w.)). LOQs were defined as the lowest point of the calibration curve, while LODs corresponded to a concentration that gives a signal/noise ratio equal to 3 for the compounds not present in the blank sample or signal intensity higher than $1x10^3$ when noise baseline was not observed. The LODs for the other compounds were estimated from compounds of the same family, taking into account their response in HRMS analysis. LODs were found to be 0.25 ng g^{-1} (d.w.) for all benzotriazole derivates, between 0.25 and 25 ng g^{-1} for benzothiazole derivates and 5 ng g^{-1} (d.w.) for benzenesulfonamide derivates. LOQs were between 0.5 and 50 ng g^{-1} (d.w.), depending on the compound, and the method was linear up to 500 ng g^{-1} (d.w.) for all compounds.

Repeatability (intra-day) and reproducibility (day-to-day) were calculated through five spiked sludge samples at two concentration levels (50 and 250 ng g^{-1} (d.w.)) and are expressed as %RSD (n=5). Repeatabilities and reproducibilities were usually lower than 10% at both concentration levels except for MeSBT, where they were slightly higher due to their low recovery yield. Absolute recoveries were determined by extracting and analysing the 5 sludge samples spiked at the two levels mentioned above (50 and 250 μg/Kg (d.w.)). The results of these spiked samples were calculated using a matrix matched calibration method with sludge samples spiked after the extraction procedure in order to calculate the absolute recoveries for each compound. Recoveries were very similar at the two levels tested and were higher than 80% for most of the compounds except ClBTR, BT and XTR, which were between 40% and 60%. Recovery of MeSBT was much lower, at around 25%.

The determination of benzotriazole, benzothiazole and benzene-sulfonamide derivates in sewage sludge samples has not received the same attention as their determination in environmental water samples, as can be seen by the few papers published involving the analysis of sludge samples. In these papers PLE was the preferred extraction technique, but only for BTRs or BTs alone. Liu et al. [6,7] determined BTRs using MeOH/CH_2Cl_2 (1:1, v/v) as an extraction solvent and obtained recoveries ranging from 66% (BTR) to 103% (XTR). Wick et al. [4] determined BTs using water/MeOH (1:1, v/v) and obtained recoveries between 53% (OHBT) and 80% (MeSBT). These results demonstrate that hot water could be a suitable extraction solvent because the recoveries obtained are similar to or better than those obtained using organic solvents, except in the case of MeSBT, for which better results were obtained with organic solvents

because it is the least polar compound.

Other extraction techniques have also been used for extracting these compounds. Zhang et al. [5] determined BTRs using solid-liquid extraction (with ethyl acetate), obtaining recoveries from 70% to 116% (the compounds are not specified in the paper), while Asimakopoulos et al. [8] determined benzotriazole and benzothiazole derivates simultaneously, obtaining recoveries of around 90% - except for MeSBT, where they were around 50% - using ultrasound assisted extraction (USAE) with water/MeOH (1:1, v/v) acidified at pH 3. We have recently developed a QuEChERS extraction [20] to determine the same compounds. The recoveries obtained (from 80% (ClBTR) to 90 % (BSA)) were similar to those obtained with PHWE, except for a few compounds for which QuEChERS was more efficient because the use of an organic solvent improves the recoveries of less polar compounds. However, the use of acetonitrile results in a higher matrix effect because more matrix components are coextracted.

As regards LODs and LOQs, the values determined using PHWE are similar to or better than those previously reported [4-8]. Compared with the QuEChERS extraction, the results by PHWE are slightly better except in the case of MeSBT because the preconcentration factor obtained in PHWE is higher than with QuEChERS and fewer matrix substances are coextracted. Moreover, PHWE offers the possibility of the semi-automation of the process during extraction which is more beneficial when a high number of samples need have to be analysed.

4 Application to sludge samples

The PHWE/SPE/LC-Orbitrap-HRMS method developed was applied to analyse ten sludge samples from five different STPs. Most of the compounds were found except for Me-*p*-TSA, Et-*p*-TSA and MeSBT. The samples were collected over two different months depending on the STP. Table 3 shows the concentrations found for the samples collected in the south-east and north-east regions. The retention time and exact mass of precursor and product ions were used to confirm the presence of these compounds following the guidelines of Commission Decision 2002/657/EC [26], and at least 4 identifications points are used depending on the compound. An HRMS extracted ion chromatogram of one of the sludge samples analysed is shown in Figure 5.

The most abundant compound found was OHBT in a wide range of concentrations from <LOQ in the STP3 and STP4 up to around 200 ng g^{-1} (d.w.) in the STP1 and STP2. The most frequently found compounds were BTRs, especially TTR isomers, which were determined in all the samples analysed. BTRs are known to be resistant to biodegradation, so this is probably the main reason why these compounds were always

Table 3. Concentration found (ng g^{-1} (d.w.)) of target compounds in sludge samples.

Compound	STP1		STP2		STP3		STP4		STP5	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
BSA	n.d.	n.d.	n.d.	n.d.	n.d.	9.8	n.d.	n.d.	n.d.	n.d.
NH_2BT	n.d.	n.d.	<LOQ	2.9	14.7	17.0	n.d.	<LOQ	n.d.	n.d.
BTR	16.0	10.2	6.5	20.4	3.5	16.1	9.1	14.0	8.7	7.0
TSA	11.8	<LOQ	n.d.	n.d.	55.5	60.2	17.8	n.d.	13.9	11.6
4TTR	28.1	51.3	14.2	23.3	8.3	19.7	56.8	28.3	33.2	35.9
5TTR	24.1	27.7	4.0	10.5	1.7	6.6	27.0	13.3	20.9	20.3
ClBTR	4.7	3.5	2.2	<LOQ	4.1	6.2	4.0	1.6	6.6	5.2
OHBT	168.7	255.4	<LOQ	<LOQ	<LOQ	<LOQ	127.8	59.1	193.6	24.2
BT	23.6	<LOQ	n.d.	n.d.	<LOQ	<LOQ	<LOQ	26.2	16.5	n.d.
XTR	1.2	0.7	3.8	0.9	4.8	10.7	1.1	1.2	3.1	2.5

determined. The higher concentration of OHBT in some samples is probably a result of the formation of degradation products from other benzothiazole derivates during anaerobic sewage treatment. The lowest concentrations of all compounds were found in the STP3, which operates with activated sludge under aerobic conditions. Very few BSA derivates were found in the samples analysed except for TSA, which was the only one frequently determined at concentrations of between <LOQ and 60.2 ng g^{-1} (d.w.). These results are in line with those previously reported for the same compounds in sewage samples from the same STPs [20]. In that paper, the most abundant compounds found were BT, TTR isomers and OHBT, which were the same as in this study.

If these results are compared with those obtained for similar samples using the QuEChERS extraction described in a previous paper [10], it can be seen that they are very similar in terms of detection frequency and concentration ranges. However, two compounds that were not detected using the QuEChERS extraction were determined using PHWE, namely NH_2BT and XTR. This is due to the lower matrix effect obtained with PHWE.

The concentrations found for BTRs and BTs were also comparable to studies from other countries, in which BTR and 5TTR were the most frequently found BTRs at levels between approximately 10 and 200 ng g^{-1} (d.w.) [5,6,8,9] and OHBT was

one of the BTs found at a higher concentration (approximately 100-300 ng g^{-1} (d.w.)) [4,8,9]. Studies on the occurrence of BSAs were not reported by other authors, but the presence of TSA in some samples has a relevant significance because the toxicity of *p*-TSA can be classified as moderate [12].

5. Conclusions

An extraction approach based on pressurised hot water for extracting different kinds of polar contaminants such as benzotriazole, benzothiazole and benzenesulfonamide derivates from sludge was developed. The analysis of sludge samples was carried out by combining PHWE with a tandem SPE clean-up followed by LC-Orbitrap-HRMS.

Absolute recoveries obtained for the whole extraction method were generally over 80% and the matrix effect was lower than 20% for most of the compounds. Repeatabilities (intra-day) and reproducibilities (day-to-day) were generally lower than 10%. LODs ranged from 0.25 to 25 ng g^{-1} (d.w.) and LOQs from 0.5 to 50 ng g^{-1} depending on the compound.

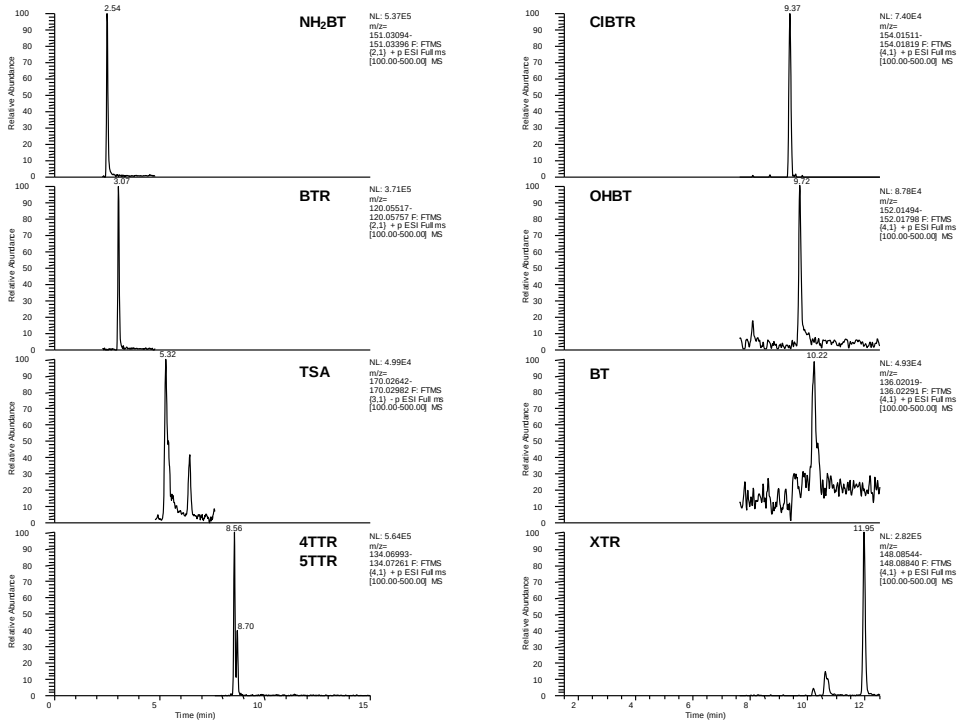

Fig. 5. A HRMS EIC of an analysed sludge sample from STP4.

The most abundant compound found was OHBT (~200 ng g^{-1} (d.w.)) and the most frequently determined compounds were BTRs. Some differences in the occurrence of the compounds studied between different sludge treatments were also observed. Using the PHWE/SPE/LC-Orbitrap-HRMS method developed, NH_2BT could be determined which was not possible by using the QuEChERS based method previously developed.

Acknowledgements

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3.3.4. Discussion of results

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The main achievement of the first study was the development of a sensitive SPE/LC-MS/MS method for the simultaneous determination of benzotriazoles, benzothiazoles and benzenesulfonamides, which had only previously been achieved using two-dimensional gas chromatography [1]. However, after the publication of this study, this has also achieved using one-dimensional gas chromatography [2] in less than 17 min, which is a similar analysis time to that obtained by LC. However, *ortho*-toluenesulfonamide was not included in this study. As previously discussed, the determination of these compounds started some years ago but it is still focus of interest for the scientific community because of the high exposure of the environment to these classes of chemical compounds. There are two main limitations on the determination of these compounds in previously reported methods and, in this study, they have been overcome satisfactorily. The first of these limitations was the determination of the isomeric species such as 4- and 5-methyl-1-H-benzotriazole because, in most of the previously reported studies, this was skipped. However, their individual determination is necessary for risk assessment studies because it has been proven that these compounds undergo different degradation ratios during sewage treatment [3]. The same applies for *ortho*- and *para*-toluenesulfonamide, for which different behaviour has been observed during sewage treatment [4-6]. To obtain their separation, a ternary mobile phase composition was found to be the best option because different selectivity was observed for tolyltriazoles and toluenesulfonamides when acetonitrile and methanol were used. The second limitation regards the quantification method used, because, due the ubiquity of these compounds in water samples, matrix-matched calibrations cannot be used and isotopically labelled compounds only exist for 1-H-benzotriazole, benzenesulfonamide and *para*-toluenesulfonamide, all of which elute at the beginning of the chromatogram. Therefore, a tandem SPE based on Oasis HLB followed by a clean-up step with a Florisil cartridge allowed sample extracts to be obtained in which the matrix effect was less than 20% for all of the compounds, both in surface waters and sewage samples. Thus, an external standard calibration is used to quantify the compounds determined in the samples.

During the development of the method for sludge samples, the chromatographic separation by means of the gradient elution profile was slightly modified to reduce the matrix effect that occurs due to the coeluting matrix components. In addition, an Orbitrap-HRMS analyser was used for the determination of these compounds in sewage sludge and similar instrumental LODs and LOQs to MS/MS were obtained.

In the case of QuEChERS extraction, its applicability has been demonstrated for sludge samples, which may extend their use in environmental analysis. In the study, the three most common buffers for QuEChERS (non-buffered, citrate and acetate) were tested and similar results were obtained for all of them. Moreover, different dispersive solid-phase extraction clean-up (dSPE) sorbents were tested, and among them, Z-sep$^+$ was chosen. For PHWE, the main result is the reduction in the matrix effect by means of performing an SPE clean-up after PHWE. The use of water allows the resulting extract to be directly loaded into the SPE cartridge, which reduces the total analysis time per sample. Overall, PHWE provided better LODs for the determination of benzotriazoles, benzothiazoles and benzenesulfonamides in sludge samples than QuEChERS. However, the QuEChERS method is faster and cheaper than PHWE and does not require sophisticated equipment.

The developed methods were applied for the analysis of surface water samples from different rivers, influent and effluent sewage samples and sewage sludge from different STPs. In this case, some of the STPs include tertiary treatments and tertiary effluent samples were also analysed to increase knowledge on the removal of these compounds. Thus, all of the compounds were determined in environmental waters but the highest concentrations determined corresponded to influent sewage samples, in which 1-H-benzotriazole was determined at concentrations around 3,000 ng/L. As observed from the results presented, the concentrations of these compounds were only slightly reduced during secondary treatment, because it has been observed that some of these compounds are resistant to biodegradation and, due to their polarity, their principal fate is not in sewage sludge. With respect to tertiary treatments, only the one based on ultra-filtration membrane treatments (Vila-Seca/Salou STP) seems to be effective in the removal of these compounds but more studies are necessary to confirm this. All of this information is presented in Figure 3.3.1 which shows the total amount of compounds, expressed as a sum of the individual concentrations determined in the different samples from STPs and treatments.

As regards sewage sludge samples, all of the compounds studied were found, except *N*-methyl- and *N*-ethyl-*para*-toluenesulfonamide, which were not detected. The presence of these compounds in sewage sludge can probably be explained by the large amounts introduced every day into sewage and their partial removal during sewage treatment. Since the samples analysed by both methods are from the same STPs (they were not taken at the same time), similar results were found in terms of the determined concentration of these compounds. The most abundant compound

determined was 2-hydroxybenzothiazole and the most frequently determined compounds were 1-H-benzotriazole and tolyltriazoles. These studies expand knowledge on the occurrence of benzotriazoles, benzothiazoles and benzenesulfonamides in sewage sludge because very few studies have reported concentrations for most of these compounds in this matrix.

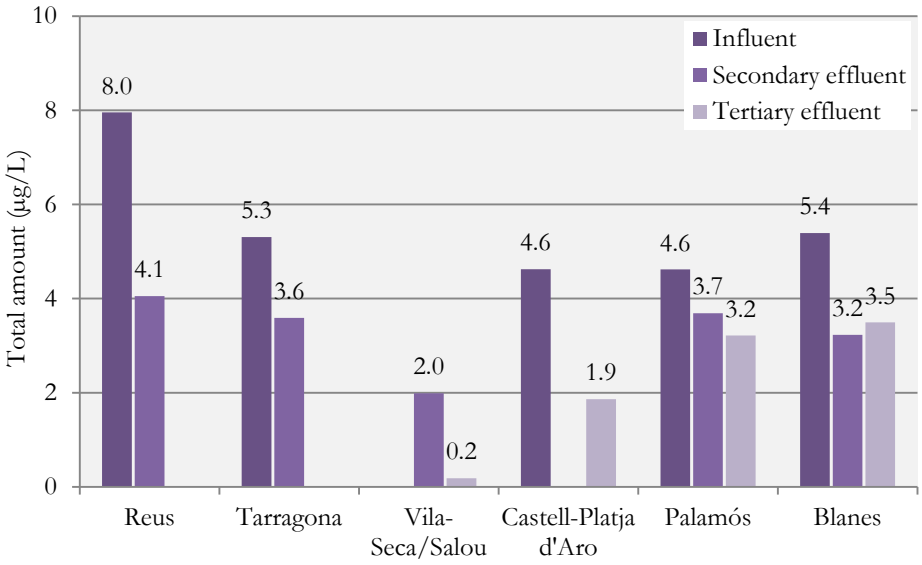

Figure 3.3.1. Total compound concentration (µg/L) in influent, secondary effluent and tertiary effluent sewage from the STPs investigated.

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3.4. Characterisation of fullerene aggregates in aqueous environmental samples

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In this section, the on-line coupling of asymmetrical flow field-flow fractionation (AF4) with a LTQ-Orbitrap-HRMS analyser for the determination and characterisation of fullerene aggregates in environmental water samples is presented for the first time. The reason for proposing the use of AF4 was because fullerenes have the ability to form stable aqueous nanometer-sized aggregates (aqu/nC_{60}), which had not previously been observed for other EOCs. Thus, size determination of these aggregates in environmental waters is crucial for risk assessment studies because it has been observed that toxicity can be size dependent [1]. The studies presented in this section were developed during my four month placement in the KWR Watercycle Research Institute (Nieuwegein, the Netherlands) under the supervision of Prof. Dr. Pim de Voogt.

The coupling of AF4 with HRMS can provide the low LODs required to use AF4 as a reliable technique for size determination at environmental concentrations of fullerenes. To date, previous studies either focused on determining the total concentration of fullerenes [2-4] by LC-based methods or on their size characterisation by different techniques. However, a method that provides both concentration and size information for fullerene aggregates has not previously been proposed. Moreover, the studies reporting size information of aggregates have only been performed in lab-scale experiments at concentrations far above real levels in the environment. For the coupling, APPI interface was selected based on the previous experience of KWR in the determination of these compounds by LC-HRMS methods [5] and previously published papers [6,7]. As mentioned earlier, the reason for using HRMS as the detection technique was to obtain LODs suitable for environmental concentrations of these EOCs because only one paper has proposed a reliable AF4 method for fullerene fractionation followed by off-line LC-MS for the quantification of the fractions obtained after AF4 [8]. However, this approach has not yet been applied to environmental samples and size information is also not obtained unless a size dependent detector such as MALS or DLS is used.

To overcome the limitation of size determination if a specific detector is not used, a more in-depth optimisation of AF4 fractionation for three (functionalised) fullerenes is attempted in the second study, with the aim of proposing a method that allows the size determination of fullerene aggregates based on fractionation time.

Lastly, river water samples and influent and effluent sewage from the Netherlands have been analysed using the proposed AF4-(APPI)HRMS method. KWR has already analysed some of these samples by LC-HRMS to quantify the

total amount of fullerenes, but they have never been analysed using AF4 for the size determination of fullerenes.

The results of these studies have been published in the *Journal of Chromatography A (2014) doi: 10.1016/j.chroma.2014.06.068* and submitted to *Analytical Chemistry.*

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3.4.1. Asymmetrical flow field-flow fractionation hyphenated to Orbitrap high resolution mass spectrometry for the determination of (functionalised) aqueous fullerene aggregates

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ANALYTICAL METHODS FOR THE DETERMINATION AND EVALUATION OF EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL WATERS AND SLUDGE.
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ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION HYPHENATED TO ORBITRAP HIGH RESOLUTION MASS SPECTROMETRY FOR THE DETERMINATION OF (FUNCTIONALISED) AQUEOUS FULLERENE AGGREGATES

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Abstract

In this short communication we report on the technical implementations of coupling an asymmetric flow field-flow fractionation (AF4) instrument to a high resolution mass spectrometer (Orbitrap) using an atmospheric photoionisation interface. This will allow for the first time on-line identification of different fullerenes in aqueous samples after their aggregates have been fractionated in the FFF channel. Quality parameters such as limits of detection (LODs), limits of quantification (LOQs) or linear range were evaluated and they were in the range of hundreds ng/L for LODs and LOQs and the detector response was linear in the range tested (up to ~ 20 μg/L). The low detection and quantification limits make this technique useful for future environmental or ecotoxicology studies in which low concentration levels are expected for fullerenes and common on-line detectors such as UV or MALS do not have enough sensitivity and selectivity.

Keywords: *Orbitrap; Field-flow fractionation; Hyphenation; Fullerenes; Nanoparticles; APPI.*

1 Introduction

Interest in nanomaterial-related applications is growing due to their novel and unique characteristics compared to "normal scale" materials [1-3]. It can therefore be assumed that nanomaterials including nanoparticles (NP) are emitted into the environment [4]. To assess the environmental risks of NPs, the development of techniques to measure and characterise them in natural environments is a priority issue [5].

Field-flow fractionation (FFF) [6], especially the asymmetrical flow version (AF4), is one of the most promising particle separation techniques that can – especially in combination with different online detectors - be used for characterisation of NPs and colloids [7,8]. However, the lack of sensitivity of many detectors commonly used, such as UV or light scattering devices, limits its use under environmentally relevant conditions [7]. Inorganic NPs, such as gold and silver NPs, can be characterised and measured at environmental concentrations by hyphenation of AF4 to an ICP-MS [9]. Carbon-based NPs, such as fullerenes [10] cannot be characterised using this combination. Several methods have been developed for the determination of concentrations of fullerenes in environmental matrices e.g., LC-UV [11], or LC-MS [12-14] using atmospheric pressure ionisation [15-17], but information about the size of their aggregates in water cannot be obtained as they need to be extracted from the aqueous phase. Information on the aggregate size is, however, crucial as the mobility and deposition of fullerenes in the aquatic environment strongly depends on this characteristic [18-20]. Therefore, up to now samples had to be analysed twice, once by using FFF to receive information on the size of the aggregates and then by using MS to determine the concentration and type of fullerene. Now it is possible to analyse each size fraction. Hence, one can see e.g. if compound A can only be found in size fractions < 50 nm and compound B in fractions > 50 nm. This was not possible with MALS or UV detectors.

It should be mentioned that fullerene clusters are destroyed during ionisation in the MS and size information cannot be obtained by use of APPI-MS alone. FFF is necessary.

To improve, shorten and ease the analysis of samples we suggest coupling AF4 to HRMS (accurate mass). In the present study AF4 was hyphenated with an Orbitrap-HRMS in order to combine particle/aggregate separation with sensitive concentration detection of three different fullerenes. The description of the technical implementation will not only allow further development of the FFF method but might also open a door to the analysis of other organic particles and aggregates. To the best of our knowledge a combination of FFF, APPI and Orbitrap-HRMS has never been reported on before. The

general feasibility of coupling FFF to a MS has been demonstrated before for ICP-MS [21-23] and ESI-MS/MS [24].

2 Experimental

2.1 Reagents and standards

C_{60}-(purity>99.9%) was purchased from Materials and Electrochemical Research Corporation (Tucson, AZ, USA). [6,6]-phenyl-C_{61} butyric acid methyl ester ([60]PCBM) (purity>99%) and [6,6]-(bis)phenyl-C_{61} butyric acid methyl ester ([60]bisPCBM) (purity>99.5%) were purchased from Solenne B.V. (Groningen, The Netherlands). Toluene (ultraresidue analyse grade) and anisole were obtained from J.T. Baker (Boom, Meppel, The Netherlands) and ultrapure water (resistivity > 18 MΩ) was obtained from a Milli-Q water purification system (Millipore, Amsterdam, The Netherlands). Milli-Q-water used as carrier liquid in the FFF was filtered through 0.1 μM membrane filters (Postnova Analytics GmbH, Landsberg, Germany) prior to entering the FFF channel.
The individual aqueous fullerenes suspensions (aqu/nC60) were prepared by extended stirring [25]. 10 mg of each compound was placed in a glass bottle containing 500 mL of Milli-Q water and they were stirred in the dark for more than one month at 25ºC. The exact concentration of the aqueous solution (filtered through 0.45 μm regenerated cellulose (RC) to remove larger particles) was determined by liquid-liquid extraction followed by LC-APPI-HRMS.

2.2 Asymmetrical Flow Field-Flow Fractionation

A Postnova AF2000 system (Postnova Analytics GmbH, Landsberg, Germany) was used. The AF4 module was coupled to a UV-detector (Shimadzu) and a Multi Angle Light Scattering detector (Postnova) The AF4 trapezoidal channel was 27.5 cm long from tip to tip, the height of the spacer was 250 μm and the permeable wall consisted of a 10 kDa RC membrane (Postnova). The carrier liquid was Milli-Q water and the injection volume was set to 100 μL using an autosampler device (Postnova). The fractionation conditions are summarised in table 1.

2.3 APPI-LTQ-Orbitrap

A hybrid LTQ Orbitrap (Thermo Electron) equipped with an atmospheric pressure photoionisation (APPI) interface (Thermo Electron) which uses a Syagen PhotoMate VUV Krypton lamp (20 eV) was employed for HRMS measurements. To optimise the MS conditions, the stock aqu/nC_{60} solution was infused in the source with a syringe pump using AF4 flow conditions. Toluene was used as a dopant and introduced in the auxiliary gas. The exact mass 720.00055 m/z $[C_{60}]^{\bullet-}$ corresponding to the molecular ion of C_{60} was monitored in full-scan in FTMS analyser at a resolution of 30,000

Table 1. Selected parameters for AF4-APPI-LTQ-Orbitrap method.

AF4 conditions	
Membrane:	Regenerated cellulose 10 kDa
Carrier liquid:	Milli-Q water
Spacer thickness:	250 μm
Detector flow:	0.1 mL/min
Split flow:	0.5 mL/min
Cross flow:	1.2 mL/min (0-12 min); 1.2-0 mL/min (12-15 min, exp. 0.2); 0 mL/min (15-20 min)
Injection time:	5 min
Injection volume:	100 μL
Injection flow:	0.2 mL/min
Focusing flow:	1.2 mL/min
LTQ-Orbitrap conditions	
Interface:	(-)APPI
Probe position:	C, 0, 0.75 μm
Capillary temperature:	350 ºC
Vaporiser temperature:	500 ºC
Sheath gas:	20 AU
Auxiliary gas:	10 AU
Sweep gas:	0 AU
Dopant:	Toluene
Dopant flow:	0.1 mL/min
Capillary voltage:	-20 V
Tube lens:	-200 V

FWHM over a mass-range of 300-1,300 Da. The optimal parameters are summarised in Table 1.

3 Results and discussion

3.1 AF4 optimisation

Information explaining the principle of FFF can be found elsewhere [6,26-28]. Generally, the optimisation of the different parameters involved in AF4 aims at the separation of monodisperse components resulting in distinct signals for each size fraction. However, the size distribution of aqu/nC_{60} solutions is polydisperse and consequently a broad signal in the fractogram is obtained [17]. Therefore, AF4 parameters were optimised to enhance the MS response but maintaining proper fractionation of fullerene aggregates which was assessed using MALS data obtained by analysing stock solutions of

aqu/nC_{60}. The carrier liquid is a limitation for coupling AF4 to MS because the latter should not be used with non-volatile electrolytes and surfactants commonly used in AF4 [29]. Ultrapure water was selected as carrier liquid because it is compatible with the MS interface and its use has been suggested before for fullerene analysis with AF4 [17].

The ratio between cross flow (F_c) and outlet flow (F_{out}) and their absolute values were optimised to separate the void peak from the analyte peak and to minimise the analysis time. Three different ratios were tested (1, 2 and 3) using a constant outlet flow of 0.8 mL/min. A ratio of 2 was selected as it results in good separation of the fullerene aggregates from the void peak, better size distribution than a ratio of 1 and less sample dilution than a ratio of 3. Different settings for F_c and F_{out} were used (all having a ratio of 2). F_{out}=0.6 mL/min and F_c=1.2 mL/min were selected as optimal flows. Afterwards, the focusing time was optimised until the peak area was constant. The optimum value was 5 min under the aforementioned AF4 conditions.

Taking into account that MS-ionisation performance with an APPI interface is highly affected by the flow rate (see Section 3.2) an interesting option to improve the analyte response is the use of a split pump to remove the upper layer of liquid at the end of the channel (slot outlet). Using this option, the resulting response measured in the MS shows an increase for the following reasons: First, under cross flow conditions the analytes are accumulated close to the membrane and the rest of the channel is void of analytes. Therefore, the upper layer of the carrier liquid is removed in the slot outlet and preconcentration in the detector flow is achieved. Secondly, an ionisation enhancement in MS is obtained when the detector flow is lowered. Thus, a split flow of 0.5 mL/min (detector flow 0.1 mL/min) was selected based on the response increment observed in MS (Section 3.2). The enrichment factor obtained via stream splitting was 6.

Under the AF4 conditions stated above, the stock solutions of functionalised fullerenes were also analysed by AF4-UV-MALS to determine the size distribution (radius of gyration). The size distribution was very similar for the three fullerenes and the particle radius spans from about 20 nm and to approximately 80 nm. The highest signal intensity can be found for particles around 50 nm.

3.2 AF4-LTQ Orbitrap coupling and optimisation

To couple the AF4 instrument with the mass spectrometer analyser an atmospheric pressure ionisation interface (API) is necessary because the outlet flow of AF4 is a liquid. The APPI interface is the most suitable API interface for fullerenes [16,30].

The ionisation of fullerenes is enhanced using toluene as dopant. For this reason, a lab-made device to introduce toluene in the APPI ionisation chamber was constructed (Figure 1). The auxiliary gas tube was

connected to the toluene flow (pumped with an HPLC pump) using a metal "T" junction. The AF4 outlet stream was connected to the auxiliary gas inlet port in the APPI probe creating a gas phase dopant delivery system.

Without this device it is almost impossible to introduce toluene to the aqueous effluent from the AF4, due to their liquid-phase immiscibility. Also, the AF4 instrument operates at low pressures (<15 bar). If the toluene is mixed with the aqueous outlet of AF4, this results in an increase of pressure.

Moreover, the introduction into the APPI probe of two immiscible solvents can result in a poor stability of the ionisation. Mixing toluene and water in the gas phase does not lead to an increase of the AF4 system pressure while the ionisation under APPI conditions is enhanced.

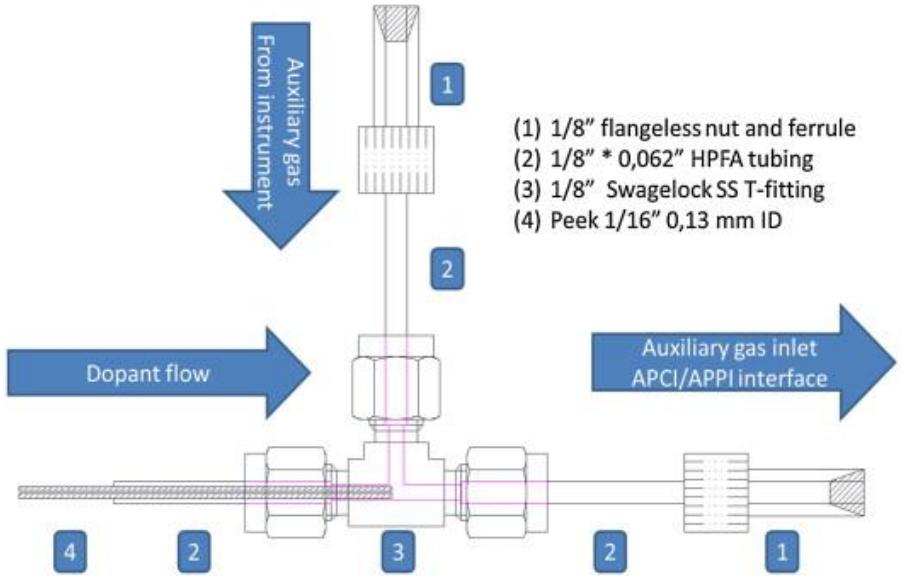

Fig. 1. Scheme of the gas-phase dopant device for coupling AF4 to HRMS using an APPI source.

For the optimisation of the APPI interface, the aqu/nC_{60} stock solution was infused (10 µL/min) into the probe, together with an AF4 flow rate (0.1 mL/min of Milli-Q water) by a "T" junction under the aforementioned conditions. The initial parameters of the interface were selected based on our previous experience and were as follows. Capillary temperature 350 ºC, vaporiser temperature 500 ºC, sheath gas 50 AU, auxiliary gas 25 AU, sweep gas 2 AU, tube lens -200 V, capillary voltage -20 V and toluene flow rate at 50 µL/min. The mass of $[C_{60}]^{\bullet-}$ (720.00055 m/z) was monitored. Different parameters were

optimised taking into account signal intensity and signal stability. Thus, the probe position (horizontal (-1 to +1), vertical (B, C or D) and axial (0.5 to 2 μm)), capillary and vaporiser temperature (from 350 to 500 ºC), sheath (from 10 to 100 AU), auxiliary (from 5 to 25 AU) and sweep gas (from 0 to 10 AU) and capillary (-5 to -120 V) and tube lens voltage (-10 to -250 V) were tested. Two different dopants (toluene and anisole (5% (v/v) in toluene) and their flow rates (10 to 200 μL/min) were tested. The optimum parameters are listed in Table 1.

First, the probe position was adjusted and the best results were obtained at position C (vertical), 0 (horizontal) and 0.75 μm (axial). The latter was the most important parameter and a closer position between the probe and lamp enhances the ionisation. Different vaporiser temperatures were tested (Figure 2) The best result was obtained at 500 ºC due to the best vaporisation of fullerene under water flow conditions and the thermal stability of this kind of compounds. Capillary temperature was changed but no differences in signal intensity were observed and therefore, it was maintained at 350 ºC. Next, the gas flow rates were optimised. A lower sheath gas flow rate results in an increase of ionisation but less spray stability was also observed. 20 AU was selected for the sheath gas flow rate. The same effect was observed for the auxiliary gas flow rate and therefore it was kept at 10 AU. The sweep gas was turned off because its use reduces the number of ionised molecules which can enter to the analyser. Furthermore, it was observed that a lower capillary voltage and a higher tube lens improve the signal intensity for fullerenes. Dopant flow rate was optimised under these conditions (Figure 2). A flow rate below 75 μL/min reduces the ionisation. At higher flow rates, an ionisation enhancement was not observed as the ionisation chamber atmosphere becomes sufficiently saturated with dopant molecules. In addition, a dopant solution consisting of a 5 % (v/v) anisole in toluene was tested. For some compounds, the use of anisole can result in a significant increase in the ionisation [31]. However, no differences between both dopants were observed. Therefore, 100 μL/min of pure toluene was selected. Moreover, APPI/APCI dual mode ionisation was tested, but less ionisation efficiency was observed.

Additionally, the effect of flow rate on ionisation efficiency was checked and optimised. The lower the flow rate, the higher the sensitivity of the APPI interface (Figure 2).For this reason a split of the flow of the AF4 outlet stream is necessary since low flow rates are not the most suitable option for AF4. Better resolution and efficiency are obtained under high flow conditions [26]. The highest ionisation efficiency was obtained at 0.1 mL/min of AF4 detector flow and then a split flow of 0.5 mL/min (to waste) and a detector flow of 0.1 mL/min (to MS) were used for the fractionation (Section 3.1).

3.3 Mass spectral characterisation

The ionisation mechanisms in negative (fullerenes ionise as $[M]^{\bullet-}$) mode are not properly understood, but probably the fullerenes were ionised via an electron capture mechanism [32,33] enhanced by the use of toluene. The effect of water in APPI ionisation is not well-studied and even less in negative ionisation. Nonetheless, the proposed ionisation mechanisms in negative mode via electron capture suggest that the use of water as mobile phase does not have effect on the ionisation performance as the proton affinity of analyte and solvent are not involved in the ionisation reaction.

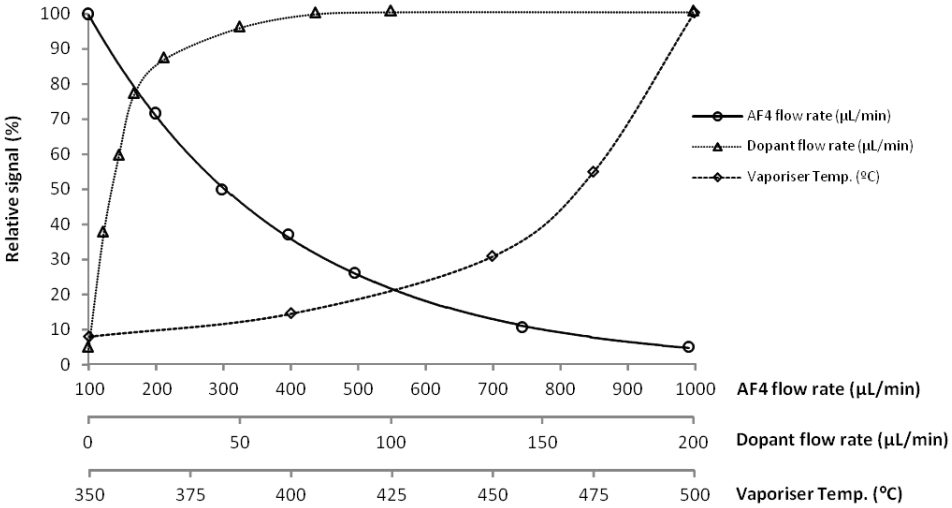

Fig. 2. Absolute response values normalised to the highest signal observed during the optimisation of flow rate, dopant flow rate and vaporiser temperature in APPI for C_{60}.

The mass spectra of fullerenes are dominated by the $[M]^{\bullet-}$ and their corresponding ^{13}C isotopic pattern (Figure 3). In addition, the oxidised adducts $[M+O]^{\bullet-}$ are also observed and for functionalised fullerenes, a small in-source fragmentation is observed (less than 5%) resulting in a weak signal of the $[C_{60}]^{\bullet-}$ ion in HRMS spectra. Figure 3 also shows that the size distribution of C_{60} (m/z 720) is the broadest one. This information could not have been deduced from MALS or UV fractograms.

3.4 Method validation

Linear range, limit of quantification and detection for C_{60}, [60]PCBM and [60]bisPCBM aqueous fullerene aggregates were validated. These

parameters were calculated by injecting 100 µL of aqueous standard solutions prepared from the aqueous stock solutions. The results are presented in Table 2, expressed as both mass amount injected and concentration (µg/L) because the injection volume is not a limiting factor in AF4 due to the focusing step.

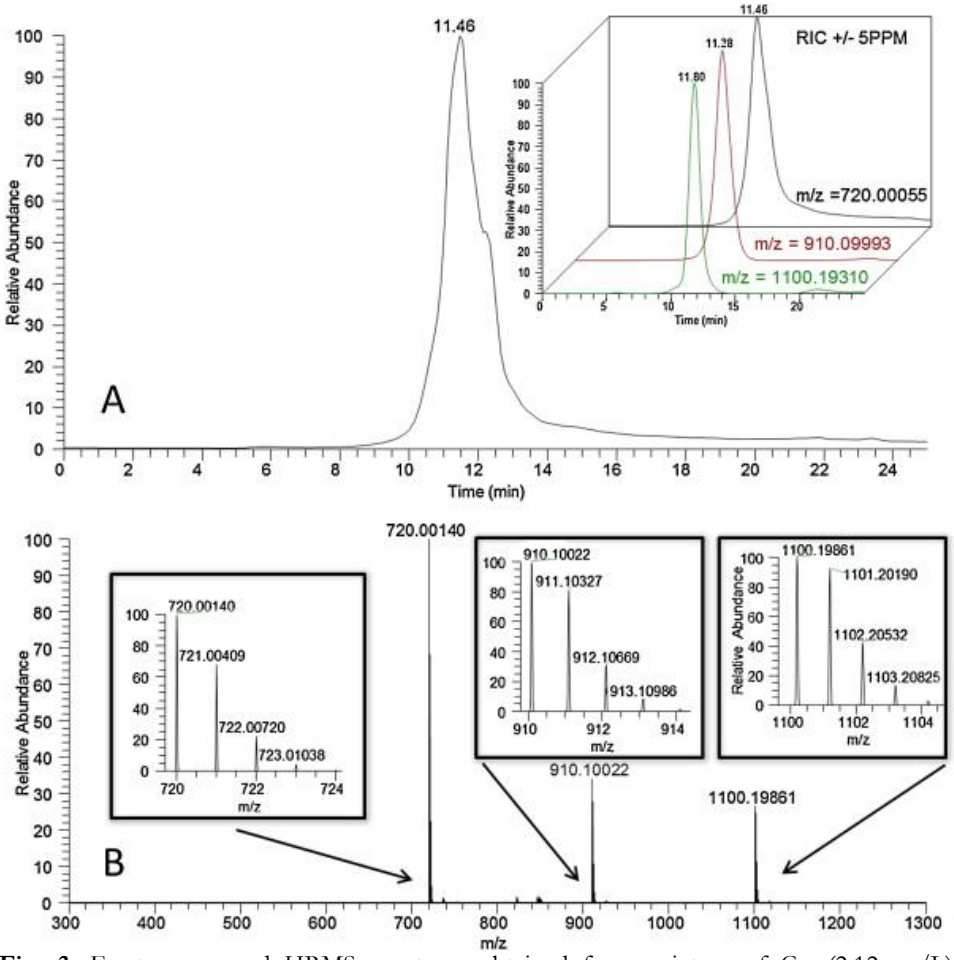

Fig. 3. Fractograms and HRMS spectrum obtained for a mixture of C_{60} (2.12 µg/L), [60]PCBM (0.88 µg/L) and [60]bisPCBM (0.66 µg/L) aqueous suspensions by AF4-APPI-LTQ Orbitrap method.

The linear range was between the LOQ and around 20 µg/L. The determination coefficient (r^2) was higher than 0.998 for all compounds. The LOQs were defined as the lowest point of the calibration curve and were between 0.3 and 0.8 µg/L. LODs corresponded to a signal/noise

ratio better than 3 and were between 0.1 and 0.4 μg/L. The repeatability of the method was assessed by injecting 5 replicates of a standard solution and the %RSD was lower than 4% for peak area variation and lower than 0.4% for the retention time at peak maximum.

Table 2. Validation parameters obtained with AF4-APPI-LTQ-Orbitrap method.

Compound	LOD		LOQ		Linear range		r^2
	μg/L	pg	μg/L	pg	μg/L	pg	
C_{60}	0.42	42	0.85	85	LOQ-21	LOQ-2120	0.9992
[60]PCBM	0.18	18	0.35	35	LOQ-18	LOQ-1770	0.9989
[60]bisPCBM	0.13	13	0.26	26	LOQ-22	LOQ-2202	0.9989

4 Conclusions

The coupling of AF4 to a LTQ Orbitrap MS using an APPI interface for the determination of aqueous (functionalised) fullerenes aggregates was successfully accomplished. The use of the slot outlet to reduce the flow to the detector and the gas-phase dopant device were two of the most important requirements. The former does not only reduce the flow to the MS, but also increases the signal intensity. Quality parameters such as LODs, LOQs or linear range were evaluated and they were in the range of hundreds ng/L and the detector response was linear in the range tested (up to ~ 20 μg/L). The low detection and quantification limits make this technique useful for future environmental or ecotoxicology studies in which low concentration levels are expected for fullerenes. Common on-line detectors such as UV,or MALS do not have enough sensitivity and selectivity. Due to the successful coupling of the FFF to an Orbitrap HRMS it is now possible to develop methods for the analysis of fullerenes in various aqueous samples at environmentally relevant conditions.

Acknowledgments

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3.4.2. Size and concentration determination of (functionalised) fullerenes in surface water and sewage matrices using asymmetrical flow field-flow fractionation coupled to accurate mass spectrometry: method development and validation

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SIZE AND CONCENTRATION DETERMINATION OF (FUNCTIONALISED) FULLERENES IN SURFACE WATER AND SEWAGE MATRICES USING ASYMMETRICAL FLOW FIELD-FLOW FRACTIONATION COUPLED TO ACCURATE MASS SPECTROMETRY: METHOD DEVELOPMENT AND VALIDATION

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Abstract

In order to assess the environmental risks of a compound it is imperative to have suitable and reliable techniques for its determination in environmental matrices. In this paper we report on the optimisation of a recently introduced field flow fractionation method that allows the determination of different fullerenes and their concentrations in different size fractions by coupling it on-line to Orbitrap HRMS. A 0.05% NH_4OH solution in water was identified as the best carrier liquid for the analysis of the three different aqueous fullerene suspensions (C_{60}, [6,6]-phenyl-C_{61} butyric acid methyl ester and [6,6]-(bis)phenyl-C_{61} butyric acid methyl ester). The MALS data received after employing the ammonia solution was consistent with both the theory and calibration using well defined Au and latex nanoparticles. The LODs obtained using Orbitrap HRMS detection were 0.1 μg/L which are significantly better than the LODs obtained by using UV (20 μg/L) and MALS detectors (5 μg/L). Environmental samples (river water and sewage) were spiked with fullerenes and the fractograms obtained for these samples revealed that the matrix does affect the size of fullerene aggregates significantly. The information obtained about their size and behaviour in environmental samples can be useful for the risk assessment of these particles.

Keywords: *Field flow fractionation; Nanoparticles; Ffullerenes; APPI; Hyphenation; HRMS.*

1 Introduction

Nanoparticles (NPs) are by definition 1 to 100 nm in size in at least one dimension. They display unique chemical and physical properties such as high strength, large surface/area ratio, thermal stability, low permeability and high conductivity [1-5]. For this reason, nanoparticles have been applied in personal care products [2], drug delivery systems [3] and solar cells [4]. In the near future, it is expected that NPs will also be applied in chemotherapy, drug delivery and labelling of food pathogens [1,2]. The estimated revenue for nanotechnology and nanomaterials in consumer products in the year 2015 is around five trillion dollars [3]. This growing interest in nanotechnology applications and products led to consider NPs as environmental contaminants [1,2, 5]. Meanwhile, several papers report on the presence of various NPs in the environment [6-10].

NPs can be subdivided into inorganic and carbon-based (organic) compounds. The latter category includes the exclusively carbon-based fullerenes. Apart from naturally occurring fullerenes, these nanoparticles are produced in amounts of 1,500 tonnes per year [11]. Therefore, also the OECD placed them on the list of environmentally relevant nano-materials [12].

Due to an anticipated rising production level, fullerenes can find their way into the environment via various routes and therefore can be expected in the air, water, soil and sediment [13]. For rivers [14], industrial effluent [14] and sewage water [15] this has already been shown. In rivers and industrial effluent concentrations up to 130 ng/L were found and in sewage water the concentrations were often significantly above 1 μg/L. In these studies the fullerenes were analysed after extraction from the aqueous matrix. This method allows for the determination of the total concentration of fullerenes. This approach does not enable the determination of the fullerene concentrations in different size fractions nor does it enable to get information on the size of fullerene aggregates in water. However, the tendency of fullerenes to form stable nanometer-sized clusters is an important characteristic of these molecules. Without this ability their solubility would be less than 10^{-11} mg/L in water. The moment these aggregates are formed the solubility is several orders of magnitude greater (up to mg/L). As size dependent toxicity has been demonstrated for other nanoparticles [16], knowledge about the size distribution of the aggregates is crucial information for risk assessment.

To be able to measure the fullerene aggregates or to determine in which size fraction the fullerenes accumulate, samples need to be analysed directly without any pre-treatment that could result in their disintegration. A technique that allows to do so is flow field fractionation (FFF) in combination

with a light scattering techniques, such as multi angle light scattering (MALS).

During the last years, field-flow fractionation techniques (specially asymmetrical flow field-flow fractionation (AF4)) have arisen in laboratories to be used in different fields [6,17-21]. The first experiments of flow field-flow fractionation were conducted by Giddings et al. in 1976 [22] but their posterior use was very limited because of a lack of reliable instruments. FFF is a size separation technique similar to size-exclusion chromatography (SEC) but without the use of a packed column and a wider size range than SEC. The fractionation principle of FFF is based on applying a perpendicular field to main parabolic flow in an open flat channel (a detailed explanation of FFF principle is given in several papers and books [10,20,23-28]). In fact, the retention time is related to the diffusion coefficient of the particle which in turn can be linked to their radius (Stokes-Einstein equation).

As FFF alone does not yield any information on the particles (except for the size calculated from the retention time), a detector is needed. Most commonly UV, ICP-MS, ICP-OES or as mentioned before MALS detectors are used. However, for measuring organic compounds at environ-mentally relevant concentrations these detector are not suitable as they either cannot detect organic particles (ICP) or they are not sensitive enough (UV, MALS) [29,30]. Neither of these detectors is able to characterise the fullerenes to boot.

Issacson et al. proposed the use of AF4 with off line LC-MS analysis for the size distribution determination of aqu/nC_{60} at environmental concentrations but it has not been done yet [31]. In an earlier paper we explained the experimental setup needed to analyse fullerenes in environmental samples with FFF coupled to an Orbitrap MS with an APPI. The high flow rates needed for AF4, as compared with liquid chromatography, makes online coupling of AF4 to MS difficult therefore we used a low flow rate taking advantage of a split pump that allows to divert analyte-free solution away from the detector. As far as we know only one other paper reported on online MS detection. However, they used hollow-fiber FFF (HF5) for the analysis of proteins [21]. The coupling of HF5 to MS is more feasible than other FFF techniques because of the lower flow rates required for the fractionation (below to 400 μL/min).

In this paper we report on the optimisation of the FFF method to analyse fullerenes in environmental samples. The fullerenes aggregates were separated in the FFF channel and subsequently measured by MS using an atmospheric pressure photo ionisation interface (APPI), as this proved to be the most suitable technique for fullerene analysis. Size determination is achieved by (I) measuring the fullerenes at a concentration high enough for the MALS detector and (II) by using

calibration compounds (Au nanoparticle and latex particles). We propose the use of a different eluent than MilliQ water, as we can demonstrate that in this eluent the fullerenes size does not coincide with the theory nor does it overlap with the size of other nanoparticles, that were used for calibration purposes.

2 Experimental

2.1 Reagents and standards

The chemical standard C_{60} fullerene (purity>99.9%) was purchased from Materials and Electrochemical Research Corporation (Tucson, AZ, USA). [6,6]-phenyl-C_{61} butyric acid methyl ester ([60]PCBM) (purity>99%) and [6,6]-(bis)phenyl-C_{61} butyric acid methyl ester ([60]bisPCBM) (purity>99.5%) were purchased from Solenne B.V. (Groningen, The Netherlands). Toluene (ultraresidue analyse grade) and anisole were obtained from J.T. Baker (Boom, Meppel, The Netherlands), NaCl from Sigma-Aldrich (Steinhem, Germany) and ultrapure water (resistivity > 18 MΩ) was obtained from a Milli-Q water purification system (Millipore, Amsterdam, The Netherlands).
The individual stock solutions of fullerenes (500 mg/L) and a mixture stock solution (40 mg/L) were prepared in toluene and were used for quantification purposes by LC-HRMS method.
The individual aqueous fullerenes suspensions were prepared by extended stirring method [32]. Around 10 mg of each compound was weighted and placed in a glass bottle containing 500 mL of Milli-Q water and they were stirred in the dark for more than one month at 25ºC. The exact concentration of the aqueous solution was determined by liquid-liquid extraction method and LC-HRMS.

2.2 Sampling

Surface water samples were taken from the river Drentsche Aa. 24 h flow dependent composite samples of sewage water (influent and effluent) were taken from a sewage treatment plant with a catchment area of 15 km^2 also covering a high density motorway network. In an earlier study the concentration of C_{60} in the influent was found to be 19 ng/l.

2.3 Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) was used to quantify the total amount of aqueous fullerene suspension used for optimisation purposes. LLE extraction was done by mixing 2 mL of aqueous suspension (filtered by 0.45 μm regenerated cellulose (RC) syringe filter) with 2 mL of 10% (w/v) NaCl solution in Milli-Q water and 2 mL of toluene in a glass vial. The solution was shaken at 150 rpm for 1 h and afterwards, an aliquot of the organic layer was directly analysed by LC-HRMS method.
For higher aqueous volumes, which are obtained during recovery experiments, the same ratio (2:1) between aqueous solution and

toluene was used but the sodium chloride was directly dissolved in the aqueous fraction to obtain a concentration of 5% (w/v) in NaCl. An aliquot of the organic layer was concentrated to less than 1 mL using an automated blow-down apparatus (Barkey, Germany) and adjusted to 1 mL with toluene by weight and analysed by LC-HRMS method.

2.4 Ultrasonic Assisted Extraction

Ultrasonic assisted extraction (UAE) was used for the extraction of the fullerenes from the AF4 membranes (10 kDa regenerated cellulose (RC) membrane and 10kDa PES (polyethersulfone) from Postnova) during recovery experiments. The membranes were dried overnight in an oven at 50ºC, cut in small pieces and placed in an Erlenmeyer flask with 25 mL of toluene. The membranes were extracted in an ultrasonic bath for 1 h, the toluene extract was concentrated to less than 1 mL using an automated blow-down apparatus and adjusted to 1 mL with toluene by weight and analysed by LC-HRMS method.

2.5 LC-(Orbitrap)HRMS analysis

The LC-HRMS method used for quantification of the total amount of aqueous suspensions was developed and validated in a previous paper [5]. The chromatographic system was a Surveyor HPLC from Thermo Electron (Bremen, Germany) and it was coupled to a hybrid LTQ Orbitrap mass spectrometer using an atmospheric pressure photoionisation interface (APPI).
The chromatographic column used (250 x 2.0 mm; 5 μm) was a Cosmosil Buckyprep (Nacalai Tesque, Japan) with a pyrenylpropyl bonded silica-based stationary phase. This column was specially designed for fullerene analysis by isocratic elution with pure toluene.

2.6 AF4-UV-MALS analysis

A Postnova AF2000 system (Postnova Analytics GmbH, Landsberg, Germany) was used for Asymmetrical Flow Field-Flow Fractionation (AF4) experiments. The AF4 module was coupled on-line to an UV detector (Shimadzu) followed by a Multi Angle Light Scattering detector (Postnova). The AF4 trapezoidal channel was 27.5 cm long from tip to tip, three different spacers (190, 250 350 μm) were tested and the permeable wall consisted of either 10 kDa regenerated cellulose (RC) membrane or 10kDa PES (polyethersulfone) (Postnova). For the optimised programme the 190 μm spacer and the RC membrane were chosen. The carrier liquid used for the MS experiments was Milli-Q water with 0.05% NH_4OH (pH ~ 9) and the injection volume for optimisation experiments was set at 100 μL using an autosampler device (Postnova).
The optimised FFF programme was as follows. The injection and focusing period was done for 5 min with a 0.2 m/L injection flow, 1.4 mL/min focusing flow and 1.5 mL/min cross

flow. The fractionation programme consisted of a constant cross flow for 3 min, followed by programmed cross flow decay (power 0.2) for 12 min and a 5 min period in which the cross flow was set at 0 mL/min. During all the experiment (injection, focusing and fractionation) the detector flow rate was set at 0.1 mL/ and the slot pump, which removes the upper layer (which does not contain analytes) of the carrier liquid at the end of the channel, was set at 0.5 m/L. Thus, the effective outlet flow during fractionation was 0.6 mL/min.

The UV detector was set at 280 and 330 nm for fullerene analysis and the MALS instrument contains 21 detectors which were used to calculate the diameter of aqu/nC_{60} aggregates. A refractive index increment (dn/dc) of 0.165 mL/g and random coil calculation were used to determine the size of aggregates using the software provided by Postnova (AF2000 control version 1.2.0.16).

2.7 AF4-(Orbitrap)HRMS analysis

The AF4 module and conditions were the same as those described in Section 2.6. For the on-line coupling with a hybrid LTQ Orbitrap (Thermo Electron), an atmospheric pressure photoionisation interface (Thermo Electron) with a SyagenPhotoMate VUV Krypton lamp (20 eV) was used with a special lab-made device to introduce the dopant flow through to the auxiliary gas port. The outlet flow from the AF4 channel was connected to the normal inlet in the APPI probe and an HPLC pump was used to introduce the toluene via the auxiliary gas by means of gas-phase dopant delivery device.

To optimise HRMS measurements, the stock aqu/nC_{60} solution was infused in the source with a syringe pump connected under AF4 flow conditions. The exact mass 720.00055 m/z $[C_{60}]^{\bullet-}$ corresponding to the molecular ion of C_{60} was monitored in full-scan at a resolution of 30,000 FWHM over a mass-range of 300-1,300 Da with the microscans set to 10. The optimised values thus obtained were -20 V for capillary voltage, -200 V for tube lens, 350 ºC for capillary temperature, 500 ºC for vaporiser temperature, 20 AU for sheath gas and 10 AU for auxiliary gas. The probe position was set at position C (vertical), 0 (side-to-side) and 0.75 μm (front-to-rear). The dopant toluene was infused at a flow rate of 100 μL/min.

The identification and confirmation of target analytes was done following the guidelines of the European Directive 2002/657/EC [33] using the isotopic mass corresponding to one atom of ^{13}C. 4 identification points (IP) were obtained for confirmation purposes.

3 Results and discussion

3.1 AF4 optimisation

The asymmetrical flow field-flow fractionation principle is accurately described in several papers [10,20,23-28]. Briefly, the theory for FFF is based on classical physical laws such

as the Brownian motion or laminar flow. Separation of particles of different sizes is achieved by applying an external field perpendicular to the parabolic flow in the separation channel. Hence, separation is not obtained by interaction between the mobile phase and a stationary phase – as it would be the case for liquid chromatography – but by interaction of the particle with the external field. Different parameters can be adjusted to optimise the separation (carrier liquid, cross flow, detector flow, fractionation mode, spacer thickness, nature of membrane, injection time and focusing time). Several papers describe the effects of changes made to these parameters [31,34,35], however to the best of our knowledge the optimisation of the fullerene cluster separation using FFF coupled to an Orbitrap HRMS has never been described before.

Due to the polydispere nature of aqueous C_{60} colloids suspensions, the result of the separation process will be a broad peak [31] or signal rather than distinct signals as can be expected for monodisperse particles, such as silver or gold [35,36]. In this paper we focus on improvement of the response of the analyte in order to develop a method for the analysis of environmental waters in which the concentration of C_{60} is in the order of a few ng/L.

Carrier liquid

It is well known that the carrier liquid has an important influence on the fractionation performance as demonstrated by the large number of published papers employing both symmetrical and asymmetrical flow field-flow fractionation in which different electrolytes and surfactants are used for different kinds of particles [9,28,37].The reason for the use of electrolytes and surfactants is the reduction of electrostatic repulsion between colloids (stabilisation of the colloidal solution) and reduction of the colloid-membrane interaction. For fullerenes eluents other than ultra pure water have not been mentioned in literature thus far [31]. Although the application of a mass spectrometer renders non-volatile additives unsuitable, for the optimisation of the FFF separation without the MS connected also non-volatile additives are considered.

Ultrapure water, 1 mM Na_2BO_7, SDS 0.01% (w/v), $NaHCO_3$ 0.01% (w/v), HCOOH 0.01% (v/v) and NH_4OH 0.05% (v/v) were tested using a spacer thickness of 250 μm and constant cross flow conditions. For SDS, $NaHCO_3$ and HCOOH solutions no UV or MALS signal for the fullerenes was observed. The aggregation of fullerenes is highly dependent of the ionic strength and carrier liquids at acidic pH also increases the formation of larger clusters as a result of the neutralisation of their negatively charged surface. When ultrapure water or two basic solutions (Na_2BO_7 and NH_4OH, pH ~ 9) are used, fractionation of the fullerene aggregates is observed. The area obtained and the average radius

across the peak with the three carrier liquids was the same, which indicates that these solutions do not affect the aggregation.

However, the fractograms of the basic solutions and ultra-pure water were very different in terms of retention time as can be seen in Figure 1 for the example of pure water and NH_4OH. The retention time and the size distribution were as expected according to the theory for the basic solutions and match previously reported size distributions. The retention time, however, was much shorter for ultra-pure water than would be expected for a constant cross-flow fractionation.

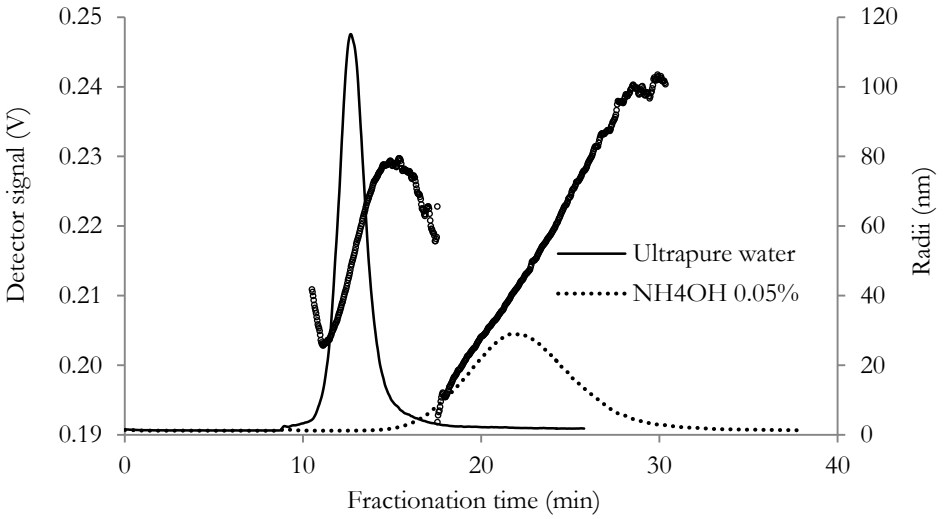

Fig. 1. Fractograms and radius size (MALS) obtained for a stock solution of aqu/nC_{60} using ultrapure water and NH_4OH 0.05% as carrier liquid under constant cross flow conditions.

A possible explanation could be the stabilisation of the negative charge of the fullerene aggregates. This might lead to less repulsion with the membrane surface and hence a longer elution time. However, there is no substantial evidence for this assumption. Also, one could wonder why the smaller particles should be affected by this. If the assumption is right they should be furthest away from the membrane and undergo no repulsion with the same. Therefore, more experiments are needed to understand the mechanism behind this phenomenon. Taking into account that non-volatile electrolytes cannot be used in MS, NH_4OH 0.05% (v/v) was favoured over Na_2BO_7.

Spacer thickness and cross flow optimisation

The spacer thickness (or channel height) plays an important role in the retention time because under the

same flow conditions the retention time is directly proportional to the square of the channel height. Reduction of the analysis time can be achieved by employing a smaller spacer. However, the efficacy of fractionation is also decreased and less resolution between two monodisperse compounds is obtained. Since aqu/nC_{60} are polydisperse, the loss in separation efficacy is less significant. Moreover, the reduction of spacer thickness increases the sensitivity as the analytes are less diluted during fractionation.

Three different spacers were tested (190, 250 and 350 μm) in combination with different cross flows (0.6, 0.9, 1.2, 1.5 and 1.8 mL/min). The spacer thickness and cross flow were chosen in order to obtain a complete separation between the injection peak and the beginning of the peak of aqu/nC_{60}. For the 190 μm spacer, at least 1.2 mL/min (a ratio of 2 of cross flow/channel flow) is necessary. For the other spacers 0.6 mL/min results in a sufficient separation but also results in longer retention times. Therefore, the spacer of 190 μm was selected for a further optimisation of the cross flow elution profile. To reduce the analysis time, the cross flow decay was optimised. The best fractionation was obtained using an initial cross flow of 1.5 mL/min which is kept constant during the first three minutes of the elution. Afterwards, cross flow decay using a power function (n=0.2) for the duration of 12 min was applied. With this cross flow profile, the smaller aqu/nC_{60} aggregates are sufficiently separated from the injection peak and the bigger ones do not need more than 10 minutes of time to elute.

Injection time and focusing

The sample injection and relaxation (focusing step) is a required step for FFF techniques as during this process the particles will accumulate close to the membrane. The focusing time was optimised by a systematic increase from 1 to 15 min until no further improvement was observed. The retention factor (ratio between times of peak maximum and the injection peak), size distribution across the peak and peak area were used to assess the optimum time. Peak splitting which is result of an incomplete relaxation was not observed in the range tested.

The retention factor and the peak area were the same for the entire focusing time range tested which indicate that losses of the smallest particles through to the membrane or irreversible accumulation of particles on the membrane does not occurred. For focusing times of 7.5 min or longer, the observed range of radii started at 100 nm and increased until 160 nm while at short focusing times the radius size started at 20 nm and it was increased until 160 nm. This suggests that a long focusing time results in the further agglomeration of smaller aqu/nC_{60} particles. Based on fractionation time and size distribution, a total time of 5 min for injection and focusing time were

selected. The injection and focusing (relaxation) times were optimised for an injection volume of 100 µL. As it takes 0.5 min to inject the sample at a flow rate of 0.2 mL/min, the optimum focusing time is 4.5 min. If higher injection volumes would be used, it is mandatory to adjust this parameter according to the sample volume. e.g., for a 1 mL sample a 5 min injection time plus 4.5 min relaxation is necessary.

Membrane selection and recoveries

Membrane selection is a crucial issue in flow field-flow fractionations techniques because some authors report that irreversible adsorption or deposition of analytes into the membrane may reduce the recovery during fractionation [20,28,31]. During sample injection and focusing step, the analytes are concentrated and driven to a specific point on the membrane and where the deposition takes place. However, it does not have any effect on the fractionation performance, only some authors reported that the first injections using a fresh membrane could result in weaker signals due to strong sample adsorption. After this, the membrane at the focusing point becomes saturated with a layer of compounds and no differences are observed for the next injections. To check if the type of membrane has an effect on the recoveries, two different membranes were tested. The first was a 10 kDa regenerated cellulose (RC) membrane and the other was a 10 kDa polyethersulfone (PES) membrane. For recovery experiments, five consecutive injections of 100 µL of a stock solution of aqu/nC_{60} (131 µg/L) were done with a fresh membrane under the above mentioned optimised AF4 conditions. The split pump was not used for these experiments and therefore the detector flow was set to 0.6 mL/min to reproduce the same fractionation conditions. The different fractions that were collected for mass balance calculations included: (I) the detector flow fraction, which contains the aqueous fraction to the detector; (II) the waste fraction, which is the cross flow that contains the fraction of analytes that pass through to the membrane pores; and (III) the membrane, which contains the irreversibly deposited analytes. The two liquid fractions were analysed by LLE and LC-HRMS and the membrane was dried in an oven at 50 ºC and analysed by UAE and LC-HRMS.

The results of the mass balance calculations for both membranes are shown in Table 1. The results obtained for both membranes are similar and merely around 2% of the analyte is irreversibly adsorbed to the membrane. However, it seems that the RC membrane is less permeable for aqu/nC_{60} aggregates based on the lower concentration found (<LOQ; 12 pg_{inj}) in the waste fraction of RC membrane as compared to PES membrane with the same cut-off (10 kDa). Nonetheless, both membranes provide a high recovery based on detector fraction but the recovery for the RC membrane (95%) was higher

than for the PES membrane (89%). These results are higher than those reported previously by the same kind of membranes by Isaacson et al. [31] who reported similar recoveries for PES membrane (79%) but less for RC membrane (73%). In addition, the fractogram obtained with both membranes was evaluated in terms of peak shape, peak area, retention time, repeatability and size distribution by MALS detector.

Table 1. Recoveries (%) obtained in each fraction collected in five consecutive injections of 100 μL of aqu/nC_{60} sock solution (131μg/L) for regenerated cellulose (RC) and polyethersulfone (PES) membranes.

Fraction collected	Recovery (%)	
	RC	**PES**
Membrane	2.3	1.7
Detector	95.3	85.3
Waste	<1%	2.2
Total	97.6	89.2

The results of both membranes are comparable. However, the retention times with the PES membrane were higher and the peak shape were better than with RC membrane, probably due to the interactions between fullerenes and the aromatic rings of the PES membrane resulting in a little increase in their retention time. The relative standard deviation (%RSD) of the fractionation time at peak maximum was lower with the RC membrane (0.3% RSD, n=5) than PES membrane (1.2% RSD, n=5).
With regard to peak area, less area is observed after the first injection using a fresh PES membrane compared with the consecutive injections in which the area remains constant (3% RSD, n=4) due to the aforementioned effect of the analyte deposition during injection and focusing. This effect is not observed in the RC membrane in which the area of the five consecutive injections remains equal (3% RSD, n=5). These small differences in the area are probably due to the inherent error of the autosampler device.
The size distribution obtained with MALS were the same for both membranes, starting around 20 nm of radii and growing up to 160 nm for aqu/nC_{60} aggregates. The average size along the peak was 82 nm when using a RC membrane and 80 nm with a PES membrane. Only a little drift in radii versus time was observed between both membranes due to the slightly higher retention observed for fullerenes when employing PES membrane. Based on these results, a RC membrane was selected for the fractionation of aqu/nC_{60} aggregates.

3.2 Determination of the hydrodynamic radius

The determination of the hydrodynamic radius is the most

important information provided by AF4 and it can be derived mathematically from the retention time when several parameters such as channel thickness, geometrical void volume or position of focusing point, are known [28]. One should realize, however, that most of the equations contain approximations and are based on constant cross flows and therefore, they cannot be used for fractionation under cross flow decay. Thus, an external calibration using two different kinds of nanoparticles to determine the radius from retention time is proposed because at low "environmental" concentrations of fullerene aggregates the MALS detector cannot be used. Mono-dispersed Gold NPs stabilized with PVP (2.5, 5, 10, 20, 30, 35 and 45 nm of radius) were used for the smaller radii and Polystyrene Latex NPs (50 and 230 nm) for bigger ones. Standards of each size were injected individually and the retention time at peak maximum (in MALS detector) was used for calibration. To corroborate the applicability of this calibration to fullerenes, the gyration radii obtained by MALS for aqu/nC_{60}, aqu/n[60]PCBM and aqu/n[60]bisPCBM solutions were compared to the radius provided by the supplier of Gold and Latex NPs as shown in Figure 2.

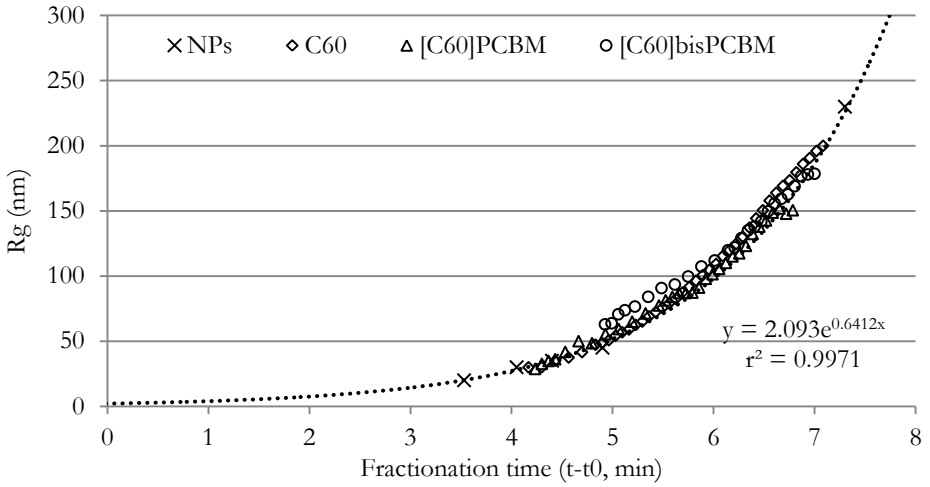

Fig. 2. Radius calibration using gold and latex NPs and data provided by MALS for aqu/nC_{60}, aqu/n[60]PCBM and aqu/n[60]bisPCBM.

The scatter points of mono-disperse NPs were fitted using an exponential regression and the radius obtained was very close to the radius provided by MALS for fullerenes aggregates. These results demonstrate the applicability of the presented size calibration method.

Moreover, the use of known size NPs allows to determine the lower particle sizes which can be separated from the void time. Only 2.5 nm (radius) gold

NPs cannot be separated, but still be detected meaning the membrane is penetrable. Therefore, the radius calibration is useful for size characterisation starting at 10 nm radius under the optimised AF4 conditions.

3.2 AF4-LTQ Orbitrap coupling and optimisation

The coupling of the AF4 instrument to the Orbitrap mass spectrometer analyser was previously reported in which an atmospheric pressure photoionisation interface and a split pump were used. Here, we described how to use the Orbitrap as an online detector for the FFF. Furthermore, we elucidate how to enhance the signal intensity by using toluene as dopant introduced into the APPI ionisation chamber by means of a lab-made device. The toluene was infused using the auxiliary gas flow entrance.

Another parameter that is important for the signal intensity in APPI is the flow rate of the FFF effluent. A low flow rate is advisable as this will increase the sensitivity of the detector. Therefore, the usage of a split flow of the AF4 outlet stream is proposed. It increases the analyte response measured by the detector because (I) the detector flow rate is lower and (II) liquid not carrying any analyte is diverted from the detector. Under cross flow conditions the analytes are compressed in a region between 1-10 μm close to the membrane. The rest of the channel is void of analytes and therefore the upper layer of carrier liquid (opposite to the membrane) can be discarded without the loss of analyte. Consequently, the split detector flow is richer in analytes and the detector signal is increased.

Different split flows (0 to 0.5 mL/min) were tested maintaining the same cross flow and channel flow and the resulting detector flow was thus decreased from 0.6 to 0.1 mL/min. As the theory says, smaller aggregates can be found predominately in the upper layers and bigger aggregates closer to the membrane. A change in the size distribution would show that smaller aggregates were removed by the split pump.

0.2 mL/min (33.3%), 0.3 mL/min (50%), 0.4 mL/min (66.7%) and 0.5 mL/min (83.3%) split flows were tested (the percentage of total flow removed is in brackets) and it was observed that the enrichment factor obtained by split flow was according to theoretical mass balance calculations as shown in Figure 3 and no differences in size distribution, according to MALS detector, were observed. Therefore, a split flow of 0.5 mL/min (detector flow 0.1 mL/min) was selected as optimum since it was fully compatible with the APPI interface and an enrichment of a factor of 6 was obtained.

Moreover, the signal areas obtained from using ultrapure water and ultrapure water with 0.05% NH_4OH showed no significant differences in the ionisation performance for the aqueous fullerenes aggregates. As reported in a previous paper, the mass spectra of fullerenes using an APPI interface in negative mode is

dominated by $[M]^{\bullet-}$ and due to the high abundance of ^{13}C isotope in fullerene molecules it was used for confirmation purposes.

3.5 Method validation

The developed method was validated in terms of linear range, limit of quantification and detection and repeatability (%RSD, n=3) for C_{60}, [60]PCBM and [60]bisPCBM aqueous fullerene aggregates. All of these parameters were calculated by injecting 100 µL of aqueous standard solutions prepared from aqueous stock solutions and the results are presented in Table 2.

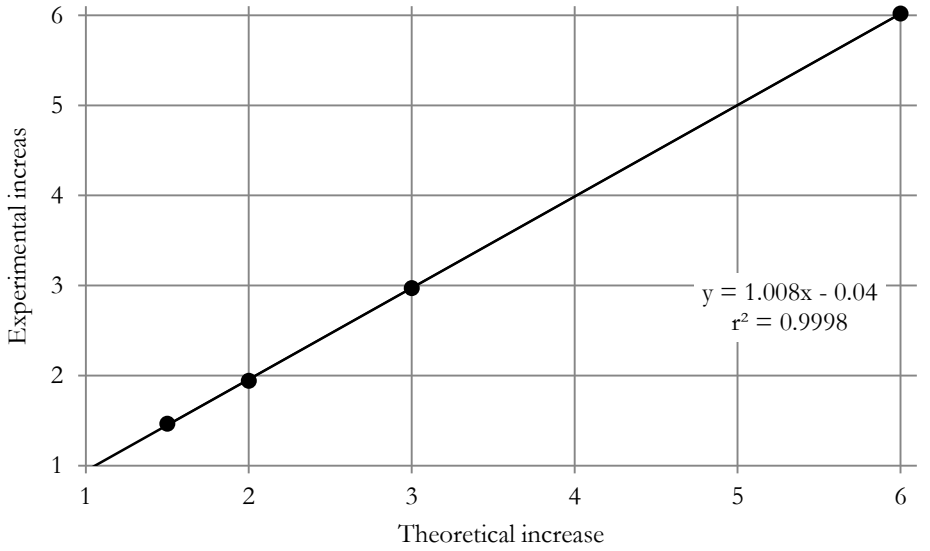

Fig. 3. Experimental increase vs. theoretical increase obtained of the UV detector response for aqu/nC_{60} by outlet split flow.

The linear range spanned from the LOQ to around 20 µg/L and the determination coefficient of the calibration line (r^2) was higher than 0.9993 for all compounds. The LOQs were defined as the lowest point of the calibration curve and were around 0.4 µg/L and the LODs corresponding to a signal/noise ratio better than 3 or a signal intensity higher than $1x10^3$ were between 0.1 and 0.2 µg/L. The repeatability of the method was determined by injecting 3 replicates of a standard solution (2.1 µg/L for C_{60}, 0.9 µg/L for [60]PCBM and 0.7 µg/L for [60]bisPCBM) and the %RSD was around 5% in peak area. The LODs using MS were much better than those obtained by UV and MALS detectors in which cases the approximated LODs were ~ 5 µg/L for MALS and ~ 20 µg/L for UV. The overlapped normalised responses for an aqu/nC60 stock solution

obtained with the aforementioned detectors are presented in figure 4. As can be seen the peak maxima of all detector signals coincide, but the response across the peak was different because the signal in MALS an UV is affected by the actual cluster, this does not hold true for the MS signal. Therefore, the response obtained by MS is more representative for an accurate quantitative determination.

Table 2. Validation parameters of AF4-APPI-LTQ Orbitrap method for the aqueous (functionalised) fullerenes agrgattes.

Compound	LOD	LOQ	Linear range	r^2	Repeatability
	μg/L	μg/L	μg/L		(%RSD, n=3)
C_{60}	0.21	0.42	LOQ-21	0.9996	5.1
[60]PCBM	0.18	0.35	LOQ-18	0.9993	6.1
[60]bisPCBM	0.13	0.26	LOQ-22	0.9994	5.2

LOD is Limit of detection
LOQ is limit of quantification

4 Application to environmental water samples

The application of the developed method to the analysis of environmental waters was done by injection of non-spiked and spiked samples of river water, influent and effluent sewage. The fractograms obtained for the different spiked samples and a standard solution at the same concentration are shown in figure 5. As can be seen, for river water the fractrogram obtained was the same as that of the standard solution which means that the aggregation of fullerenes is not affected by the natural components in this river water sample. However, when spiked sewage samples were analysed the size distribution of fullerenes is strongly affected, especially in the effluent samples analysed, and the fractionation of fullerenes starts in the void time. These results suggested that bigger clusters were disaggregated into smaller ones due to the presence of organic and inorganic matter. Studies have shown that the most stable form for colloidal clusters of fullerenes are based on icosahedral arrangements of 13 C_{60} molecules separated by water layers [38]. This is the basic aggregation of C_{60} clusters with an estimated size of 3.4 nm and then, several smaller clusters will form bigger aggregates. Therefore, the fractionation observed for sewage waters suggests that bigger aggregates degrade and hence, it can be expected that these smaller clusters appear in the void time. For effluent samples the major concentration of fullerene aggregates have a radius lower than 5 nm but a second maximum is also observed between 11 and 12 min which corresponds to a radius between 35 and 65 nm. For influent samples, the peak of aqu/nC_{60} starts

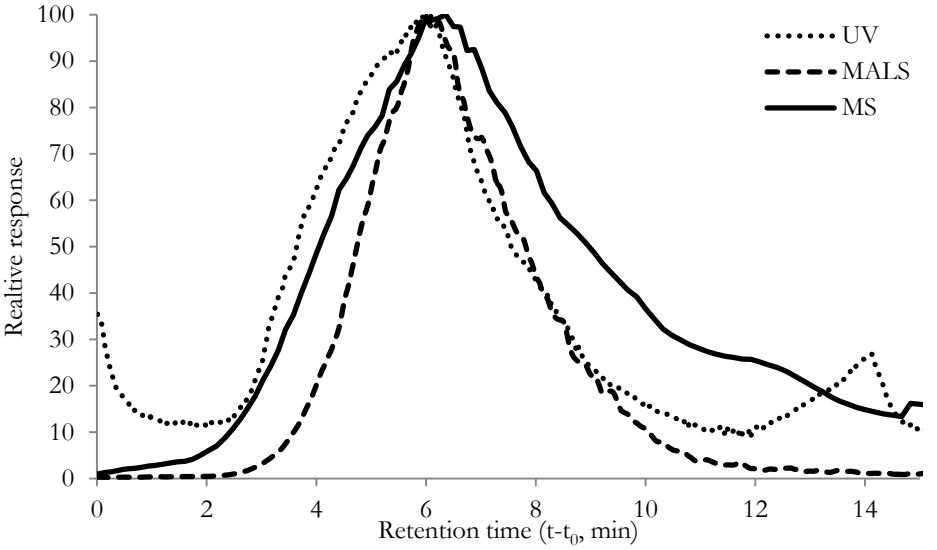

Fig. 4. Fractograms obtained for a stock solution of aqu/nC_{60} by MALS, UV and MS detectors.

after the void time and the maximum amount was found at 12.6 min which corresponds to a radius of 50 nm. The radii determined of aqu/n[60]PCBM and aqu/n[60]bisPCBM were lower than for aqu/nC_{60}, starting < 5 nm and the peak maximum observed for aqu/n[60]bisPCBM corresponds to a radius of 25 nm. For aqu/n[60]PCBM the response was more or less constant until 90 nm. For river samples, the radii for all of the functionalised fullerenes started at around 15-20 nm with a maximum around 100 nm.

With regard to non-spiked samples, no fullerene aggregates were found. Apparently, the water samples investigated in the present study do not contain fullerene derivatives above the current LOQ (few ng/L). The use of higher injection volumes (injecting 2 mL of sample equivalent to a 20-fold increase) would correspond to an estimated LOD of around 20 ng/L.

5 Conclusions

In this paper we demonstrated for the first time that field flow fractionation allows the determination of fullerene concentrations in different size fractions by using an on-line Orbitrap HRMS. The choice of solvent is crucial for the applicability of an online MS as non-volatile additives cannot not be used. Milli-Q water was excluded as suitable eluent as the data received was not in accordance with the FFF theory. A 0.05(v/v)% NH_4OH solution was found to be the best eluent for the analysis of the three different fullerene suspensions (C_{60}, [6,6]-phenyl-C_{61} butyric acid

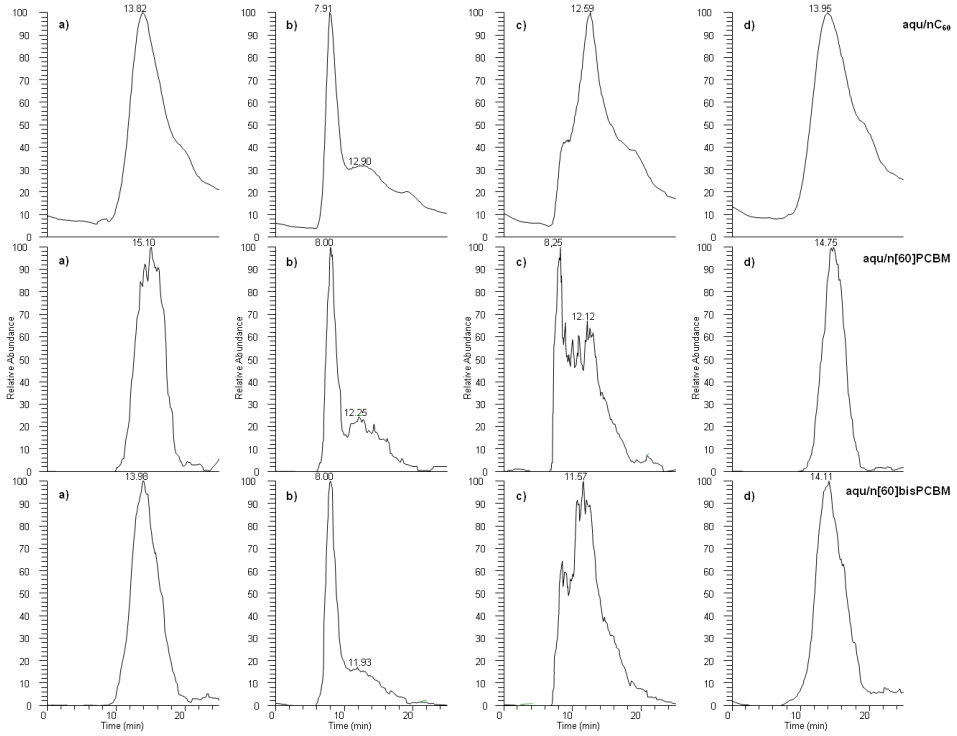

Fig. 5. Fractograms obtained by AF4-APPI-LTQ Orbitrap for: a) river water; b) effluent sewage; c) influent sewage; d) standard. The spiked concentrations of aqu/nC_{60}, aqu/n[60]PCBM and aqu/n[60]bisPCBM were 2.1, 0.88 and 0.66 μg/L, respectively, in all samples.

methyl ester ([60]PCBM) and [6,6]-(bis)phenyl-C_{61} butyric acid methyl ester ([60]bisPCBM)). The MALS data received with this eluent were consistent with the theory and with calibration using Au and latex particles. The LOD of the Orbitrap HRMS was about 0.1 μg/L and significantly better than the LOD for the UV (20 μg/L) and the MALS detector (5 μg/L) for an injection volume of 100 μL. However, these LODs can be further improved as in theory there is no limit to the amount of sample that can be injected into the FFF. Environmental samples (river and sewage water) were spiked with fullerenes and the fractograms revealed that the matrix does effect the size of the aggregates significantly. As size is an important factor when it comes to toxicity and transport, this information will be helpful to understand the impact that fullerenes can have on the environment and living organisms.

Acknowledgments

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3.4.3. Discussion of results

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These studies present the on-line coupling of AF4 with mass spectrometry (LTQ-Orbitrap) for the first time for the size and concentration determination of aqueous fullerene aggregates. The combination of these two techniques is capable of providing information about the size and concentration of these aggregates at environmental concentrations due to the higher sensitivity provided by (APPI)HRMS compared to other detection techniques previously coupled to AF4.

The most critical point was to interface both instruments because current interfaces for organic compounds are not fully compatible with usual AF4 conditions, such as flow rate or carrier liquid composition. Therefore, the fractionation of fullerene aggregates was optimised to obtain fractionation conditions that were fully compatible with available atmospheric pressure interfaces. In this case, APPI was chosen because it has been demonstrated that is the ionisation interface which provides the best sensitivity for fullerenes [1,2]. Firstly, suspensions of aqueous fullerene aggregates were made using the extended stirring method, because it seems to provide aggregates more similar to those found in environmental samples [3]. These stock solutions were used for optimisation purposes. Since fullerene fractionation using AF4 had already been reported [4], similar conditions were chosen to start the experiments. For example, ultrapure water was chosen as the carrier liquid solution. Since ionisation of fullerenes is enhanced by a dopant-assisted mechanism using toluene [2], this was introduced into the interface by the auxiliary gas inlet. As can be seen in the first study, there are many factors which affect ionisation and these were subsequently optimised. One of the major drawbacks was the low flow rates required for ionisation and so a split pump was used to reduce the flow that enters the APPI interface after fractionation.

In addition, a more in-depth optimisation of the AF4 fractionation was performed because non-ideal behaviour of aggregates was observed during fractionation. As AF4 theory states [5], fractionation time can be used to calculate the hydrodynamic radii of the particles. However, the radii obtained by theoretical calculations are far from the radii obtained using a MALS detector when ultrapure water is the carrier liquid. Moreover, fractionation does not change accordingly if the cross flow/channel flow ratio is changed, which is in contrast to the principle of FFF techniques. Therefore, several carrier liquid solutions were tested. However, if they contain salts, fullerene aggregates were not observed during fractionation, which is in line with previous knowledge of the KWR. In addition, non-volatile buffers are also not compatible with MS and, eventually, ammonia solution was found to be the only carrier liquid which provided more suitable fractionation and

was compatible with MS. However, once again, radii calculations were not in line with the theoretical basis and so an external size calibration using size-calibrated gold and latex nanoparticles was proposed for their size determination. The use of these particles was assessed by the determination of the radii of fullerene aggregates measured by MALS and interpolated to the calibration curve (radii vs. time) obtained with the calibration particles, of the radii are already well known. This calibration method has also recently been used for silver nanoparticles [6].

The optimised AF4-(APPI)LTQ-Orbitrap-HRMS method was applied to analyse river water and influent and effluent sewage samples from the Netherlands, because both studies were conducted there. In the samples analysed, no fullerene aggregates were determined by the proposed method. However, the same samples were analysed by a previously developed LC-APPI-HRMS method and only C_{60} was determined in the influent sample at a concentration of 19 ng/l. Therefore, the LODs obtained were not enough for the current concentration of this compound in this sample. However, the same samples were also analysed by injecting 2 mL of sample (20-folds increase) but C_{60} fullerenes were not detected. Studies with spiked samples were then carried out to test the developed method and their applicability was shown if these compounds are present at concentrations reported in other studies in which they were determined at concentrations up to μg/L in some cases.

This is the first study in which AF4-(APPI)HRMS has been used for the determination of fullerene aggregates in environmental aqueous samples.

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CHAPTER 4. CONCLUSIONS

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The main conclusions drawn from the studies presented in this Doctoral Thesis can be summarised as follows:

1. The methods developed in this Doctoral Thesis to determine glucocorticoids, polyether ionophores, benzotriazoles, benzothiazoles and benzenesulfonamides have limits of quantification low enough for their determination in surface water, sewage and sewage sludge samples.

2. When both ultra-high performance liquid chromatography using columns with sub-2 μm totally porous particles and liquid chromatography by using columns with superficially porous (fused-core) particles were tested, short analysis times, good resolution and high efficacy were obtained under the optimised conditions which demonstrated their applicability for future research.

3. Chromatographic separation of the isomers betamethasone and dexamethasone, and 4-methyl-1-H-benzotriazole and 5-methyl-1-H-benzotriazole, was achieved in the developed methods which allowed their individual determination in environmental samples.

4. Solid-phase extraction provided excellent recoveries for all of the compounds tested in this Thesis. Different commercially available balanced hydrophilic/hydrophobic polymeric sorbents and mixed-mode sorbents were tested during the method development, with balanced hydrophilic/hydrophobic sorbent Oasis HLB providing better recoveries in all of the studies.

5. Pressurised liquid extraction was a useful technique for extracting most of the emerging organic contaminants included in this Thesis from sludge samples. In addition, pressurised hot water extraction (PHWE) was useful for extracting benzotriazoles, benzothiazoles and benzenesulfonamides. This is an environmentally friendly extraction technique and it can be followed directly by SPE clean-up.

6. Tandem SPE consisting of an HLB cartridge for percolating the samples and a Florisil cartridge for clean-up provided excellent results in terms of recoveries and matrix effect for the determination of benzotriazoles, benzothiazoles and benzenesulfonamides in river water and sewage samples.

7. QuEChERS extraction was successfully applied for the determination of benzotriazoles, benzothiazoles and benzenesulfonamides in sludge samples for the first time and, high recoveries were obtained for most of the compounds. In addition, new horizons in terms of sample extraction procedures for sludge samples have been opened with QuEChERS.

8. Of the different strategies tested to reduce matrix effect in sludge samples, solid-phase extraction clean-up and effective chromatographic conditions are the ones which provide best results.

9. When tandem mass spectrometry was compared to high resolution mass spectrometry for the determination of glucocorticoids and polyether ionophores, similar sensitivity was obtained. However, better selectivity and confirmatory capabilities were obtained by using high resolution mass spectrometry.

10. Asymmetrical flow field-flow fractionation was successfully coupled to mass spectrometry for the determination of fullerene aggregates for the first time. Adjusting the injection volume, limits of detection suitable for the determination of these compounds in environmental samples can be obtained.

11. Asymmetrical flow field-flow fractionation for fullerene aggregates was optimised to overcome non-ideal behaviour during fractionation of these compounds. Thus, the use of ammonium hydroxide solution as carrier liquid allows the fractionation of fullerene aggregates following the AF4 principle and their size is not affected.

12. A size determination of fullerene aggregates based on an external size calibration by standardised gold and latex nanoparticles was successfully applied and the size of fullerene aggregates could to be determined from their fractionation time.

13. The results obtained for spiked samples with fullerenes show that the aggregation of fullerenes is not affected by natural components of river waters but the size distribution of fullerene aggregates is strongly affected in sewage, especially in the effluents. The radius for fullerene aggregates was

between 15 and 100 nm for river water samples and, between 35 and 65 nm for sewage samples.

14. The glucocorticoids cortisone and cortisol, the polyether ionophores monensin and narasin, the benzotriazoles 1-H-benzotriazole and tolyltriazole isomers, the benzothiazole 2-hydroxybenzothiazole and, the benzenesulfonamide *para*-toluenesulfonamide were the most abundant compounds of each group of emerging organic contaminants included in this Thesis found in the river water, sewage and sewage sludge samples analysed.

15. The presence of *N*-methyl-*para*-toluenesulfonamide in sewage and of monensin, narasin, benzenesulfonamide and *para*-toluenesulfonamide in sewage sludge samples was reported for the first time in this Thesis.

16. Tertiary treatments based on ultra-filtration membranes seem to be more effective than conventionally STP treatments for the removal of benzotriazoles, benzothiazoles and benzenesulfonamides.

17. When surface water and sewage samples from the Netherlands were analysed with the AF4-(APPI)HRMS method, fullerene aggregates were not detected.

18. The studies presented in this Thesis have further demonstrated the presence of glucocorticoids, polyether ionophores, benzotriazoles, benzothiazoles and benzenesulfonamides in environmental waters and sewage sludge samples. Because the presence of these emerging organic contaminants in the environment may have negative effects, it is important that these contaminants are taken into account when drawing up new legislation.

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APPENDIX

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Appendix I. Abbreviations used in this Doctoral Thesis.

[60]bisPCBM	[6,6]-(bis)phenyl-C_{61} butyric acid methyl ester
[60]PCBM	[6,6]-phenyl-C_{61} butyric acid methyl ester
4TTR	4-methyl-1-H-benzotriazole
5TTR	5-methyl-1-H-benzotriazole
ACN	Acetonitrile
AF4	Asymmetrical flow field-flow fractionation
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
ASE	Accelerated solvent extraction
BMS	Betamethasone
BSA	Benzenesulfonamide
BSAs	Benzenesulfonamide derivates
BT	Benzothiazole
BTR	1-H-benzotriazole
BTRs	Benzotriazole derivates
BTs	Benzothiazole derivates
cAF4	Chip asymmetrical flow field-flow fractionation
ClBTR	5-chloro-1-H-benzotriazole
COR	Cortisone
DCM	Dichloromethane
DES	Diethylstilbestrol
DLLME	Dispersive liquid-liquid microextraction
DLS	Dynamic light scattering
DMS	Dexamethasone
dSPE	Dispersive solid-phase extraction
EDC	Endocrine-disrupting chemical
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EOC	Emerging organic contaminant
ESE	Enhanced solvent extraction
ESI	Electrospray ionisation

EtmTSA	*N*-ethyl-*meta*-toluenesulfonamide
EtOAc	Ethyl acetate
EtoTSA	*N*-ethyl-*ortho*-toluenesulfonamide
EtpTSA	*N*-ethyl-*para*-toluenesulfonamide
FDA	Food and drug administration
FFF	Field-flow fractionation
FlFFF	Flow field-flow fractionation
FMS	Flumethasone
FTICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
GC	Gas chromatography
GCB	Graphitized carbon black
HCOR	Hydrocortisone
HF5	Hollow-fibre flow field-flow fractionation
HFME	Hollow-fibre microextraction
HPLC	High performance liquid chromatography
HPSE	High pressure solvent extraction
HPV	High production volume
HRMS	High resolution mass spectrometry
ICCA	International council of chemical associations
ICP	Inductively coupled plasma
ILSVA-SME	Ionic liquid supported vortex-assisted synergic microextraction
IP	Identification point
IRMS	Isotope ratio mass spectrometry
IT	Ion trap
LAS	Lasalocid acid
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LOD	Limit of detection
LOQ	Limit of quantification
MAD	Maduramicin
MAE	Microwave assisted extraction
MALDI	Matrix-assisted laser desorption ionisation

MALS	Multi-angle light scattering
MeBT	2-methylbenzothiazole
MeOH	Methanol
MepTSA	*N*-methyl-*para*-toluenesulfonamide
MeSBT	2-(methylthio)benzothiazole
MeSHBT	4-methyl-2-mercaptobenzothiazole
MON	Monensin
MorBT	2-(morpholinothio)benzothiazole
MPNL	Methylprednisolone
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl tert-butyl ether
NAR	Narasin
NH_2BT	2-aminobenzothiazole
NIG	Nigericin
NM	Nanomaterial
NP	Nanoparticle
OECD	Organisation for economic co-operation and development
OHBT	2-hydroxibenzothiazole
oTSA	*Ortho*-toluenesulfonamide
PES	Polyethersulfone
PFE	Pressurised fluid extraction
PHWE	Pressurised hot water extraction
PLE	Pressurised liquid extraction
PNL	Prednisolone
PNS	Prednisone
PSA	Primary-secondary amine
PTFE	Polytetrafluoroethylene
pTSA	*Para*-toluenesulfonamide
QqQ	Triple quadrupole
RC	Regenerated cellulose
SAL	Salinomycin
SBSE	Stir bar sorptive extraction

SDB	Styrene-divinylbenzene
SDME	Single-drop microextraction
SEC	Size-exclusion chromatography
SedFFF	Sedimentation field-flow fractionation
SHBT	2-mercaptobenzothiazole
SIM	Selected ion monitoring
SLE	Solid-liquid extraction
SO_3BT	Benzothiazole-2-sulfonic acid
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
SS-BVMME	Supramolecular solvent-based vortex-mixed microextraction
STP	Sewage treatment plant
SUPRAS	Supramolecular solvents
TACA	Triamcinolone acetonide
TBP	Tributyl phosphate
TCMTBT	2-(thiocyanomethylthio)benzothiazole
TD	Thermal desorption
ThFFF	Thermal field-flow fractionation
TOF	Time of flight
TTR	Tolyltriazole
UA-DLLME	Ultrasound-assisted dispersive liquid-liquid microextraction
UHPLC	Ultra-high performance liquid chromatography
USAE	Ultrasound assisted extraction
USEPA	United States environmental protection agency
UV	Ultraviolet
WHO	World health organization
WW	Waste water
XTR	5,6-dimethyl-1H-benzotriazole

Appendix II. List of publications.

P. Herrero, F. Borrull, E. Pocurull, R.M. Marcé, *Benzotriazoles, benzothiazoles and benzenesulfonamides in the environment: an overview of analytical methods and occurrence*, Trends Anal. Chem. (2014) (accepted) (Section 1.2.3.).

P. Herrero, F. Borrull, E. Pocurull, R.M. Marcé, *Determination of glucocorticoids in sewage and river waters by ultra-high performance liquid chromatography-tandem mass spectrometry*, J. Chromatogr. A 1224 (2012) 19-26 (Section 3.1.1.).

P. Herrero, F. Borrull, R.M. Marcé, E. Pocurull, *Pressurised liquid extraction and ultra-high performance liquid chromatography-tandem mass spectrometry to determine endogenous and synthetic glucocorticoids in sewage sludge*, Talanta 103 (2013) 186-193 (Section 3.1.2.).

P. Herrero, F. Borrull, E. Pocurull, R.M. Marcé, *Novel amide polar-embedded reversed-phase column for the fast liquid chromatography-tandem mass spectrometry method to determine polyether ionophores in environmental waters*, J. Chromatogr. A 1263 (2013) 7-13 (Section 3.2.1.).

P. Herrero, F. Borrull, R.M. Marcé, E. Pocurull, *Determination of polyether ionophores in urban sewage sludge by pressurised liquid extraction and liquid chromatography-tandem mass spectrometry. Study of different clean-up strategies*, J. Chromatogr. A 1285 (2013) 31-39 (Section 3.2.2.).

P. Herrero, N. Cortés-Francisco, F. Borrull, J. Caixach, E. Pocurull, R.M. Marcé, *Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultra-high performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks*, J. Mass Spectrom. 49 (2014) 585-596 (Section 3.2.3.).

P. Herrero, F. Borrull, E. Pocurull, R.M. Marcé, *Efficient tandem solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry method to determine polar benzotriazole, benzothiazole and benzenesulfonamide contaminants in environmental water samples*, J. Chromatogr. A 1309 (2013) 22-32 (Section 3.3.1.).

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