STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS

Daniela Salas Acosta

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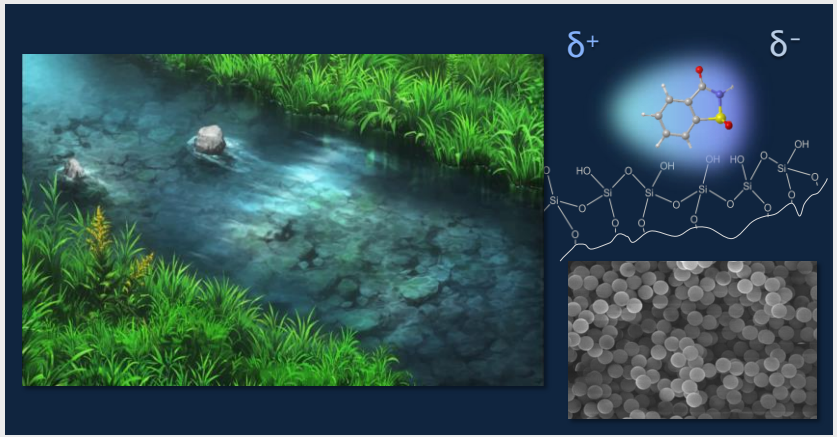

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DOCTORAL THESIS

Supervised by

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Department of Analytical Chemistry and Organic Chemistry

UNIVERSITAT ROVIRA I VIRGILI

Tarragona

2017

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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. NÚRIA FONTANALS i TORROJA, Investigadora 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: "STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS", presentada per DANIELA SALAS ACOSTA per optar al grau de Doctor per la Universitat Rovira i Virgili amb menció internacional, 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'esmentada doctoranda, i compleix els requeriments per a poder optar la menció internacional.

I, per a que consti, signem aquest document a Tarragona, 3 de gener de 2017.

Dra. Rosa Maria Marcé i Recasens

Dra. Núria Fontanals i Torroja

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ABSTRACT

Despite the efforts focused on reducing the environmental impact of modern industrialization and urbanization, contamination produced by the release of thousands of substances is still a problem to all living species of this planet. The determination of these contaminants is necessary in order to design strategies for their elimination; determination that is accomplished using selective and sensitive analytical methods capable of quantifying these compounds, which are present in complex matrices at low concentrations. In this regard, liquid chromatography (LC) coupled to mass spectrometry (MS)-based detection is the technique of choice to analyze environmental water samples. However, a significant number of the contaminants introduced into the aquatic environment have polar character and, because of these properties, they frequently exhibit problems during the sample preparation and chromatographic separation steps. Motivated by this, the aim of the present Doctoral Thesis is the application of recent techniques focused on improving the determination of polar contaminants.

One of these recent strategies is hydrophilic interaction liquid chromatography (HILIC), an LC mode developed to enhance retention and separation of polar compounds poorly retained or separated with difficulties, on conventional chromatographic modes. Similarly, mixed-mode ion-exchange polymeric sorbents are improved solid-phase extraction (SPE) materials in terms of capacity and selectivity to address limitations found for ionizable compounds. Both recent strategies are thoroughly investigated in the present Thesis, focusing on their fundamentals and retention mechanisms as well as their concrete advantages for the analysis of river and wastewater samples.

In the sections devoted to the application of HILIC, the separation of two groups of polar compounds rarely studied by this LC mode is described. HILIC was initially investigated in terms of its retention behavior for a group of iodinated X-ray contrast media, to gain insights into the influence of several chromatographic parameters on separation and the retention mechanisms involved. The effect of changing the mobile phase conditions

and the influence of the stationary phase chemistry on retention behavior was observed. In addition, the results suggested that HILIC operates under a multimodal retention mechanism with important contributions of hydrophilic partition, adsorption and hydrogen bonding.

Once proficient in the use of HILIC, the technique was coupled with high-resolution mass spectrometry (HRMS) to develop a new alternative method for the determination of a group of artificial sweeteners in river and wastewater samples. The separation achieved exhibited higher retention and better selectivity than the other few HILIC methods reported in the literature. Moreover, as HILIC is compatible with the injection of organic solutions, the sample treatment procedure was simplified by directly injecting organic extracts into the LC system.

In the other sections, mixed-mode ion-exchange polymeric sorbents were exploited in alternative applications beyond their conventional use. Although they are conceived for the selective SPE of ionic compounds, a study included herein demonstrated that they can also be used for the extraction of neutral compounds capable of developing induced dipoles. Thus, new methods were developed for the selective extraction of a group of benzotriazoles and benzothiazoles using a simple SPE procedure and achieving a reduction in matrix effect, thanks to the introduction of a washing step with methanol to eliminate neutral interferences.

The application of these materials was also broadened to promote cation- and anion-exchange interactions simultaneously, using a single SPE cartridge and following a simple protocol, instead of limiting to their conventional use of promoting one type of ion-exchange interaction. For this purpose, commercial sorbents of each type were combined for the first time to obtain positive and negative charges within the same cartridge. A new method was developed using this approach for the simultaneous extraction of acidic and basic compounds from environmental waters.

Motivated by the good results obtained when combining cation- and anion-exchangers, new mixed-mode ion-exchange polymeric sorbents were developed in-house containing zwitterionic moieties. Their SPE evaluation,

applied to a group of pharmaceuticals, illicit drugs and artificial sweeteners selected as model compounds, gave interest insights about the morphology of the materials and the interactions promoted. The most promising sorbent was very selective for both acidic and basic compounds with specific properties. The results obtained in this study are the starting point for the development of new functional materials with the potential of retaining acidic and basic compounds more efficiently in terms of capacity and selectivity.

The use of both HILIC and mixed-mode ion-exchange sorbents has proved to be valuable and promising alternatives to resolve the problems associated to polar compounds during the sample treatment step and the chromatographic separations. The results obtained here contribute to the overall knowledge of both techniques, proposing new methods for contaminants seldom studied using these approaches or innovative applications that exploit their capabilities further than the most conventional uses.

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CHAPTER 1. INTRODUCTION

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A large number of organic chemicals are currently used by modern society for medical, industrial, domestic and other purposes, which have significantly improved our quality of life. Unfortunately, several of these compounds reach and pollute the environment through several pathways. Compared to persistent organic contaminants whose accumulation in the environment and toxicity is widely known, emerging organic contaminants (EOCs) are pollutants that were previously unknown or not classified as contaminants and their occurrence in the environment has only been studied in the last few decades, including compounds developed or discovered in the environment in this period [1, 2]. The presence of EOCs in the aquatic environment, soils and air is of great concern. Therefore, their occurrence and research into associated risks to human health, as well as regulations, have been extensively reported and reviewed [1-8]. The principal sources of EOCs are anthropogenic, including industrial, domestic and hospital disposals (such as wastewaters), disposal from aquaculture activities and leachates from landfills and agricultural fields [8]. They include pharmaceuticals and personal care products (PPCPs), illicit drugs, compounds used or produced by industrial processes, veterinary products, food additives, pesticides and flame retardants, to name a few, in addition to the metabolites and/or transformation products (TPs) of these substances [3, 5].

At low levels (ng/L), several of these contaminants have an impact on the ecosystem or human health, with examples of this fact being the exposure of fish to hormones or other endocrine disruptors and of several living species to antibiotics [1]. In the case of hormones, these are naturally excreted or synthetically produced contaminants that affect the reproductive functions of fish species and they are potentially harmful for other animal species [9]. With respect to the release of antibiotics to the environment, this represents a major concern because they can promote antibiotic resistant bacteria and antibiotic resistance genes in bacteria that give rise to both infection and economic problems [10]. In general, the risks posed by continuous exposure to low levels of several of these contaminants and even their intake through drinking water are unknown but it may potentially be harmful [3].

As most EOCs are transported and end up in aquatic bodies, a lot of attention has been paid to their presence in wastewaters and surface, sea and drinking water [4, 6, 11]. Since the first European water policy was introduced in 2000 (Directive 2000/60/EC), several groups of substances have been included on the watch list of substances for monitoring throughout the European Union under environmental quality standards, with the last amendment being made as a result of Decision 2015/495/EU, of 20th March 2015, which included a total of 17 new organic compounds [8]. However, the list of EOCs that reach the aquatic environment is enormous, as most of them are only partially removed or not removed at all during treatment procedures in wastewater treatment plants, and several metabolites or TPs are produced in the different stages between their consumption and their release into the environment. Moreover, several pathways that these contaminants follow are not subjected to treatment processes (e.g. runoff from agriculture and livestock) so they reach bodies of water very easily [6].

The detection and quantification of trace levels (ng/L) of EOCs in complex matrices such as environmental waters is a challenging task, requiring selective and sensitive analytical methods. For this purpose, chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) are normally used to analyse environmental water samples [4]. Because the contaminants found in environmental waters are mostly compounds with medium to very polar and hydrophilic properties, analytical methods have undergone several improvements to address the problems associated to these properties more effectively [2]. According to Noguera-Oviedo *et al.* [1] in their very recent review, the presence of EOCs in the environment has rocketed over the last two decades because of the development of analytical instruments capable of determining polar and water-soluble compounds, as most EOCs are not suitable for analysis by GC/MS, which was the conventional technique for determining trace organic contaminants before the appearance of LC/MS.

Several properties contribute to the overall polarity of a compound, such as dipolar moment or partition coefficient, which make these solutes able to promote dipole-dipole, proton-donor, proton-acceptor, hydrogen bonding or

electrostatic interactions. The limitations related to the determination of polar compounds have been a recurrent issue throughout the development of chromatographic methods commonly used today, associated to both their separation and the extraction procedures that are used during the sample treatment. These problems and the approaches that analytical chemists have had to adopt to resolve them are described below, as well as the most recent trends to determine this kind of compounds successfully.

Current instrumental chromatographic methods are the result of the first advances made in GC instrumentation, which was coupled with MS before LC. Polar compounds are usually non-volatile and, as such, their analysis using GC frequently required previous derivatization procedures. By the time that the coupling of LC instruments with MS-based detectors was possible, the preferred LC mode was reversed-phase (RPLC), which had already replaced the normal phase mode (NPLC) after the development of new stationary phases. This "normal" mode separated the samples on polar stationary phases using nonpolar organic solvents as mobile phase, as shown in Figure 1. It was later proven that a system with opposite polarity based on a nonpolar stationary phase and aqueous-based mobile phases was more advantageous and versatile [12, 13]. NPLC exhibited slow equilibration, irreproducibility of retention times, and tailing or fronting of the peaks as a result of heterogeneity of the retention sites [14]. Moreover, several polar compounds are insoluble in the organic solvents used in NPLC as mobile phases, or they are irreversibly retained on the stationary phases.

Nowadays, RP phases are the columns most commonly used for several chromatographic applications, in particular C_{18} columns based on octadecyl ligands bonded to silica supports. Several polar compounds that were not suitable for analysis by GC or NPLC could be successfully separated by RPLC methods, but very polar compounds often exhibited lack of retention in these nonpolar phases [15, 16].

There are several chromatographic alternatives for separating polar, hydrophilic and ionizable compounds poorly retained by the RPLC mode, such as ion chromatography (IC), ion pair chromatography (IPC) and

hydrophilic interaction liquid chromatography (HILIC), which are summarized in Figure 1. IC or IPC have been used for the separation of ionizable compounds. However, the use of IC for organic ions is quite limited, being mostly used for inorganic ions. Nowadays, IC-inductively coupled plasma (ICP)-MS has been used for speciation analysis (determining different species containing a specific metal) and the study of metals (metallomics). Within the environmental field, the occurrence of species containing arsenic is of special interest. IC-electrospray ionization (ESI)/MS has also been applied to determine molecular-mass anions such as perchlorate, phosphorus oxyanions, nitrite, nitrate, haloacetic acids and oxyhalides in several matrices including geothermal water and soil samples, among others [17]. However, the application of IC-ESI/MS for organic trace analysis is limited to a few studies [17-21].

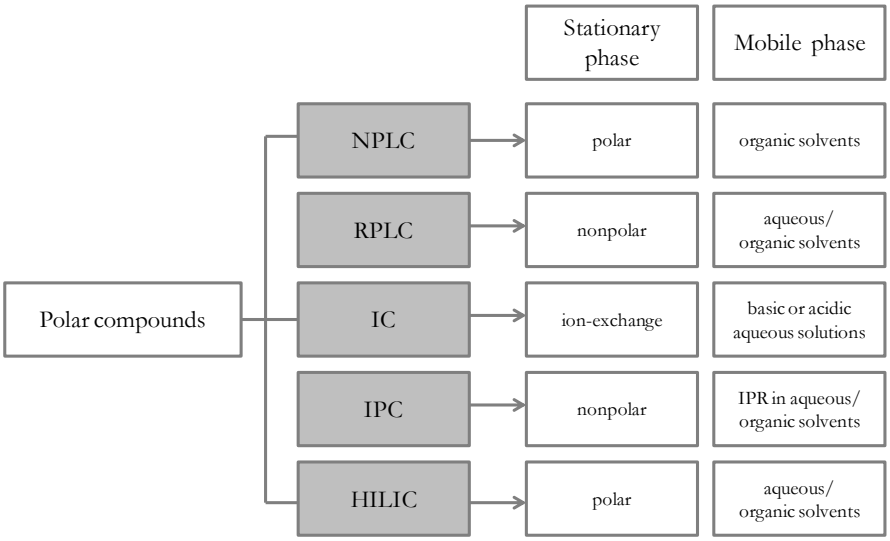

Figure 1. Different LC modes available for the separation of polar compounds.

Meanwhile, IPC has been more widely used to address the retention problems of ionic compounds, as the LC instruments and RP columns available can be applied for this purpose without major changes. In this case, the addition of an ion-pairing reagent (IPR) to the mobile phase is required with lipophilic and ionic properties capable of modifying the surface of the

stationary phase through the adsorption of the reagent via the hydrophobic side, leaving the ionic groups available for interacting with the target analytes. Hyphenation of this chromatographic mode with MS has allowed the separation and quantification of ionic organic compounds. However, the major limitation of IPC-MS methods is the lack of compatibility of several IPRs with the ion sources used in MS. The list of available reagents with volatile properties reduces the possibilities of finding a suitable IPR for the separation. Furthermore, their ion-pair interaction with the target analytes causes severe ion suppression, affecting the sensitivity of the method. Several strategies have been tested to address this problem, such as the post-column addition of reagents to pair the IPR, the use of alternative ion sources, on-line removal of the reagent using exchange columns or splitting devices. However, all of these approaches are laborious and time-consuming and many of the volatile IPRs used have displayed drawbacks in the chromatographic separation itself [22].

HILIC has recently emerged to address the limitations of the existing chromatographic modes to separate not only ionic compounds but also neutral analytes with medium to polar properties. It combines NPLC stationary phases with the aqueous mobile phases used in RPLC, with the stationary phase being relatively more polar than the mobile phase, so water is used as a stronger eluting solvent. The use of HILIC has increased over the last decade and it continues to grow thanks to the advantages that it offers, such as simplicity, improved retention, alternative selectivity and excellent compatibility with MS detectors in contrast to other chromatographic modes [14, 16, 23-25]. The improvement of polar stationary phases developed specially for this LC mode has also contributed to the growth of this new separation technique. In the present Doctoral Thesis, HILIC is one of the strategies investigated in relation to separating polar analytes of environmental relevance. With this in mind, Section 1.1. focuses on this chromatographic mode.

Regardless of the chromatographic mode used, MS-based detection is often selected because it is a sensitive, accurate and selective technique. In addition, the LC conditions used must be compatible with the ion sources used in MS.

Most methods developed to determine target polar compounds in environmental waters are based on LC-tandem mass spectrometry (MS/MS), particularly using triple quadrupole (QqQ) analyzers [26]. MS/MS emerged to overcome the limitations of single quadrupole MS in terms of selectivity and sensitivity, being capable of quantifying analytes at trace levels when working under the selected-reaction monitoring (SRM) mode. Using SRM, the molecular ion of the analyte is isolated in the first quadrupole, subjected to fragmentation in the second (which operates as a collision cell) and the product ions obtained from the parent ion are analyzed in the third. QqQ instruments are quite sensitive but sometimes exhibit limitations in terms of selectivity. For reliable confirmation of the analytes using a QqQ detector, at least two transitions from the parent compound to the product ions are needed. However, sometimes only one transition is observed. Moreover, some of these transitions are not characteristic, such as the loss of water or carbon dioxide. LC-(QqQ)MS/MS also has limitations when the simultaneous analysis of a large number of compounds is needed in a single run, because the acquisition times of the transitions narrow the number of target analytes that can be detected [26-28].

High-resolution MS instruments with time-of-flight (TOF) or Orbitrap analysers have been developed to address the limitations of selectivity in MS-based detectors. As they are capable of differencing between two different mass-to-charge (m/z) ratios up to 5 decimal places, they are the most selective detectors available today of all analytical instruments. In addition, these instruments operate in full-spectrum acquisition mode under high-resolution accurate-mass conditions, which enables the detection of thousands of target analytes or the discovery of unknown compounds [29]. When acquisition is performed as described, raw data can be retrospectively consulted in search for analytes that initially were not of interest [30].

MS instruments based on TOF analysers have been available for more than five decades and have been improved significantly over the years thanks to progress in electronics and the renewed interest in these analysers due to their suitability for pulsed laser sources [31]. In this respect, several applications in the biological and polymeric field have benefited from the

development of matrix-assisted laser desorption/ionization TOF (MALDI-TOF) instruments [32, 33]. Moreover, this type of analyser is often combined with others, with the Q-TOF hybrid analyser being one of the most successful types of hybrid instruments, along with the QqQ, achieving enhanced sensitivity compared to instruments based on single TOF [31].

The Orbitrap is one of the most recent analysers developed, based on the new concept proposed by Makarov [34, 35] and commercialized in 2005 by the Thermo Electron Corporation. This analyser is an electrostatic ion trap that uses the Fourier transform (FT) to obtain mass spectra [31]. A diagram of this analyser is shown in Figure 2 and, as can be seen, it consists of a spindle-shaped central electrode surrounded by a pair of external electrodes in the shape of a barrel.

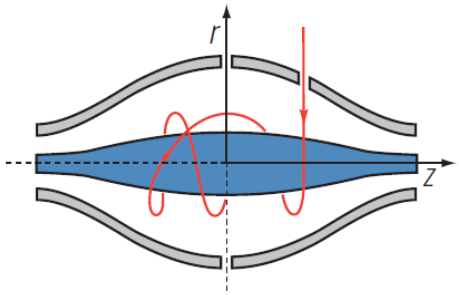

Figure 2. Diagram of the Orbitrap analyser (Thermo Fisher Scientific Exactive Operating Manual).

For the separation of ions, an electrostatic voltage is applied to the central electrode while the external one is at ground potential. The injected ions start to oscillate in the trap along the z axis in intricate spirals around the central electrode with a quadro-logarithmic potential distribution, thanks to the voltage and geometry of the trap. The frequency of the oscillation is related to mass-to-charge ratio (m/z) of the ions and the mass spectrum is obtained by the FT of the broadband current induced by the oscillating ions. With this analyser, up to a 150,000 FWHM (full width at half maximum) resolution at

m/z 600 has been achieved. Compared to a Q-TOF instrument, the resolution is significantly increased, together with the dynamic range [29, 31].

Today, Thermo Scientific has different Orbitrap-based MS systems available, including hybrids with ion traps (IT) and Q analysers, which highlight the improvement in instrumentation achieved in recent years. The Exactive or Exactive Plus Orbitrap LC-MS types are based on a single Orbitrap analyser and an optional higher-energy collisional dissociation (HCD) cell, capable of delivering high-resolution mass data at full scan acquisition and all ion fragmentation spectra. The Q Exactive Quadrupole-Orbitrap MS instrument contains a Q mass filter for precursor ion selection, while the Orbitrap Fusion Tribrid LC-MS combines Q, IT and Orbitrap analysers to maximize sensitivity and selectivity in the analysis of very complex samples [36]. In Table 1, a comparison is given between some models of these types of Orbitrap-based instruments. The performance between the instruments increases from left to right on the table, as well as the cost.

Table 1. Comparison between Orbitrap models commercialized by Thermo.

Features	**Exactive Plus (EMR)**	**Q Exactive (Plus)**	**Orbitrap Elite**	**Orbitrap Fusion**
Characteristics	Orbitrap	Q-Orbitrap	IT-Orbitrap	Q-IT-Orbitrap
Maximum resolution (FWHM) at m/z 200	140,000	140,000	340,000	500,000
Mass accuracy (ppm)	< 1	< 1	< 1	< 1
Scan rate (Hz)	12	12	4	18
Parallel Reaction Monitoring	-	yes	yes	yes

In the last few years, LC-HRMS methods have proven to be very helpful in the target, suspect and nontarget screening of environmental samples [37, 38]. Particularly, applications using Orbitrap MS instruments have been reviewed and described in different fields, such as environmental, toxicology, doping control, food analysis and several others [29, 39-41].

The type of ionization source used is as important as the analyser when coupling LC with MS-based detectors in order to obtain a good response. The techniques based on atmospheric pressure ionization, such as ESI, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), are soft ionization processes that yield molecular ions, avoiding excessive fragmentation of the analytes. In ESI sources, the formation and charge of liquid droplets are simultaneous while, in APCI, the droplets are formed first and subsequently charged before being introduced to the analyser. In APPI, a UV light source replaces the corona discharge used in APCI. Unlike ESI, APCI is more capable of ionizing low to medium polar compounds through chemical ionization processes, but it requires a certain degree of volatility and leaves remaining substances that were not efficiently ionized. In fact, the motivation for the development of APPI was to ionize these remaining molecules more effectively. For these reasons, the source most broadly used for polar compounds is ESI, which has proven to perform well with standard solvents and provide superior sensitivity [27, 42].

The matrix effect (ME) observed in these sources is one of the principal limitations associated to the analysis of complex samples. In ESI-MS instruments, this effect is observed by the suppression or enhancement of the response caused by other compounds present in the matrix [43]. There are several strategies to reduce or correct the ME observed and the selection among them must be based on the properties of the analytes and the sample, the method quantification limits (MQLs) desired and the cost of the analysis. For correction, the use of isotopically labelled internal standards is frequent, as these compounds added to the sample would undergo the same ME as the unlabelled analytes, because they have almost identical chemical and physical properties [44, 45]. However, isotopically labelled standards are very expensive, are not always commercially available for all target compounds and their use does not always successfully correct the ME.

Another strategy is the use of matrix-matched calibration, in which calibration curves are performed in the matrix of the samples instead of the pure solvent. The limitation of this approach is that it is matrix dependent and, as such, different calibration curves must be constructed for each type

of matrix analyzed [46]. In addition, several analytes are ubiquitous in the samples of interest, so finding a matrix free of analytes is not possible. When levels are low and sensitivity is good, blanks can be subtracted from the spiked samples for the construction of the matrix-matched calibration curve. However, if the levels found for the analyte are high, this approach cannot be used because the spiked sample with low levels or analytes would be indistinguishable from the blanks. In these cases, the use of external calibration is an alternative, taking into account the recoveries of the overall procedure [47]. Another easy and fast approach for reducing ME is the dilution of the sample or the extract from the sample. However, this involves increasing the detection (MDLs) and quantification (MQLs) limits of the method [48, 49].

Another particularly important strategy is the introduction of clean-up steps during the sample preparation procedures [49]. The elimination from the matrix of compounds that interfere with the ionization in the MS ion sources often leads to a reduction in the ME. Sample preparation procedures often used for this purpose can also exhibit drawbacks when dealing with polar compounds, demonstrating that the limitations and problems found for this type of analytes do not end once the chromatographic separation has been resolved.

Ideally, the purpose of the sample preparation step is to enrich the analytes of interest from the matrix of the sample in a proper state to be injected in the analytical instruments and in suitable concentrations to obtain a high response. There are several procedures to help the analytical chemist with this purpose and the selection of one methodology will depend on the state of the sample, complexity of the matrix, expected levels of the analytes, availability and costs, among other considerations.

Solid-phase extraction (SPE) is usually the preferred sample preparation technique for liquid samples, having replaced the conventional liquid-liquid extraction (LLE). This technique is based on the sorption of the analytes on a sorbent with the objective of preconcentrating them, eliminating interferences, changing solvents or increasing selectivity for the target

compounds. SPE is an exhaustive technique that aims to extract the analyte completely, in comparison to equilibrium techniques in which the partition of the analytes between the sorbent and the sample reaches equilibrium, as is the case for other SPE formats, such as solid-phase micro-extraction (SPME) and dispersive solid-phase extraction (dSPE), and other techniques like stir-bar sorptive extraction (SBSE).

A typical SPE procedure includes the conditioning of the sorbent packed in a cartridge with organic solvents and water, loading of the sample, washing steps and elution of the target analytes. Other sorptive extraction techniques share the same principle of trapping the analytes in a suitable sorbent and subsequently desorbing them in elution steps, with the differences between the techniques being the format or amount in which the sorbent is exposed to interact with the sample. In the case of SPME, the sorbent is coated onto a fused-silica fiber which can be immersed in a liquid sample or can even be exposed in the headspace of the sample if the target analytes are volatile and separated by GC. This miniaturized technique is simple, reproducible and environmentally friendly because it uses less solvent. However, it might be less sensitive. In the case of dSPE, an amount of the bulk sorbent is placed in direct contact with the sample and further separated by filtration. Another miniaturized technique is micro-extraction in packed syringe (MEPS), in which a quantity of the sorbent is placed on top of a GC syringe needle.

An alternative extraction technique is SBSE, which involves coating a sorbent onto a magnetic stir bar that is immersed in the liquid sample for a selected period of time to retain the analytes. Subsequently, the SBSE is removed from the sample and submerged in an elution solvent. In this technique, the surface of material available is greater than in SPME, improving recoveries and therefore the sensitivity of the method. However, the sorbents available for this technique are more limited in comparison to SPE and also the automatization of this procedure is more difficult [50]. Recent research has focused on developing new sorbents with polar properties for SBSE in order to enhance the retention of polar compounds [51, 52].

New sorptive techniques have recently been developed with the aim of improving capacity, simplicity and the speed of the extraction, including fabric phase sorptive extraction (FPSE) [53] and dynamic fabric phase sorptive extraction (DFPSE) [46]. Both techniques consist of fabric pieces coated with sorbents using sol-gel technologies which are either immersed in the samples or placed in a filtration assembly to percolate the sample, respectively.

Methods based on SPE and LC-MS/MS are very common when analyzing environmental waters [54, 55]. Being a mature technique, no changes in the fundamental concepts of SPE have been introduced and instrumental advances have focused on automation or miniaturization. The most active research topic in SPE and in other sorption-based extraction techniques is the development of new sorbent phases to resolve the remaining practical problems and, of course, the application of these new sorbents to relevant fields [50, 56]. Since the introduction of pre-packaged and disposable cartridges for SPE containing bonded silica sorbents with reversed-phase or normal phase properties, several sorbents have been developed to extract a wide range of compounds [55, 57]. Nowadays, a broad spectrum of materials is available, ranging from versatile sorbents capable of retaining a wide selection of compounds with different properties to highly selective materials like immunosorbents (IMSs), restricted-access materials (RAMs) or molecularly imprinted polymers (MIPs) capable of retaining specific antigens, high-molecular mass compounds or "template" analytes, respectively [58].

Nowadays, sorbents are based on porous polymeric materials developed to address the drawbacks of silica-bonded sorbents, such as instability at extreme pH value and retention problems associated to residual silanol groups, or limitations of carbon-based sorbents, such as excessive retention. The first polymeric sorbents to be widely used were macroreticular copolymers of styrene-divinylbenzene (St-DVB) which are capable of retaining compounds through hydrophobic interactions (mainly π-π interactions) and have surface areas up to 500 m^2/g. To increase sorption capacity, polymeric sorbents with high specific surface (>1,000 m^2/g) have been commercialized or developed in-house. These sorbents are

hypercrosslinked materials which are often synthesized using Friedel-Crafts reactions over existing polymers. However, several polar compounds are poorly retained in these hydrophobic sorbents and, as a consequence, polymeric sorbents with hydrophilic properties have been developed to improve their extraction [57].

These more recent sorbents combined the polymeric backbone with polar functionalities, introducing copolymerizing monomers with the desired functionalization or introducing polar groups in post-polymerization reactions [57, 58]. One of the best examples of these types of sorbents is the commercially available Oasis HLB, manufactured by Waters, which is a poly(N-vinylpyrrolidone-divinylbenzene) (PVP-DVB) copolymer that has balanced hydrophobic/hydrophilic properties [59]. This sorbent is widely used and has proven to yield high recoveries for a large number of compounds with different polarities [60-62]. Other hydrophilic sorbents modified with polar functionalities have been commercialized by Agilent and Phenomenex under the brand names Bond Elut Plexa and Strata-X, respectively. Furthermore, in-house sorbents have also been developed by some research groups which have shown better features than commercial sorbents [58].

Despite the great acceptance that has been observed for this type of sorbents, several ionizable compounds are poorly retained on these materials and, as a consequence, a new class of polymeric materials have emerged known as mixed-mode sorbents. These are synthesized combining the polymeric backbone with ionic functional groups, normally introduced to the existing hydrophobic or hydrophilic polymers [63]. These polymers represent a relatively recent strategy to address the limitations observed in terms of the extraction of ionic compounds, and the development and application of novel materials are under research.

This Doctoral Thesis focuses on approaches for solving retention and separation problems related to hydrophilic, polar and ionic compounds, as several contaminants of environmental waters have these properties. Regarding chromatographic separation, HILIC is the most recent and

promising technique and, with this in mind, the first part of this introduction presents a detailed description of the retention mechanisms, parameters affecting chromatographic separation and recent environmental applications of this chromatographic mode. The second part introduces mixed-mode ion-exchange polymeric sorbents as novel SPE materials for retaining ionic compounds, discussing the several applications found in the literature and future trends.

1.1. Hydrophilic interaction liquid chromatography

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

Chromatographic analysis using HILIC conditions dates back several decades but it was not until the landmark publication by Alpert *et al.* [64] in 1990 that the term "hydrophilic interaction chromatography" was coined to refer to this type of LC mode. As previously mentioned, it emerged in response to the lack of retention of polar and hydrophilic compounds on conventional C_8 or C_{18} reversed-phase columns, by using polar stationary phases in combination with mobile phases consisting of mixtures of water and water-miscible organic solvents [14, 65].

Precursor studies of HILIC included separation of sugars on aminopropyl silica in the presence of ACN-water and the determination of basic compounds on silica columns using reversed-phase and methanolic eluents [66, 67]. Some of these cases did not work under typical HILIC conditions, but they exhibited at least some of the mechanisms that are now considered contributory to HILIC [68]. The quality of the separations and the detailed discussion of the retention mechanisms described in the study of Alpert set the groundwork for HILIC being seen as a very promising technique. In Alpert's work, a group of peptides, nucleic acids and proteins was separated on different hydrophilic phases, revealing that higher retention was achieved at high contents of organic solvent and that selectivity was different from RP separations. He also proposed a retention mechanism based on hydrophilic partition between a water layer immobilized on the surface of the stationary phase and the mobile phase mostly organic, as illustrated in Figure 3, with contributions from ion-exchange interaction if the column had ionic functional groups [64].

1.1.1. Retention mechanisms in HILIC

Since Alpert's study, a lot of research has focused on investigating the retention mechanisms in HILIC [69-72]. Because the hydrophilicity of the compounds is related to their retention in HILIC, some studies have investigated the relationship between the retention factor (k) of model analytes and their partition coefficient (log D) [73-75]. It was soon demonstrated that correlation coefficients deviated from unity, which was attributed to the presence of additional interactions apart from hydrophilic

partition. Ionic interactions were presented as one of these additional mechanisms observed for compounds with acidic or basic properties. For instance, separations on bare silica phases showed enhanced retention for basic compounds and decreased retention for acidic compounds, which was attributed to ionic interactions between the analytes and the negatively charged silanol groups of the stationary phase [73]. Comparative studies of the retention of model compounds on a selected list of stationary phases with different functionalities concluded that specific interactions between the solutes and the surface functional groups must also be present [76]. A complex and multimodal retention mechanism was then proposed, in which hydrophilic partitioning, hydrogen bonding, adsorption on the stationary phase, electrostatic interactions and even hydrophobic interactions contribute to a greater or lesser extent to the overall retention, depending strongly on the acidic and basic properties of the analytes and the stationary phases [16].

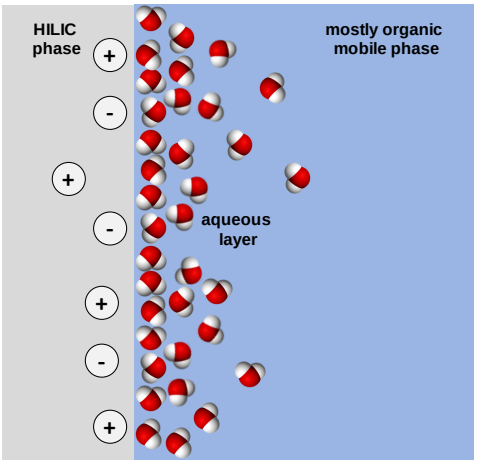

Figure 3. Diagram of the water layer formed on the surface of the HILIC stationary phase.

Several strategies have been used to study HILIC mechanisms, including chromatographic and chemometric methods. The chromatographic approach includes studying different parameters affecting separation, comparing different stationary phases and testing specific types of interactions [69]. Retention models developed for other chromatographic modes have been

applied to HILIC separations to obtain information of the predominance of a particular retention mechanism. In many studies [14, 69-71, 77], the relative contributions of partition and adsorption have been assessed by evaluating the adjustment of retention data to the equation that describe both mechanisms. The description for a mechanism based on partition is provided by the empirical equation:

$$(1) \qquad logk = logk_W - S\varphi$$

where k_W is the hypothetical retention factor when the mobile phase contains only the weaker solvent (organic solvent in the case of HILIC) and φ is the volume fraction of the strong eluent (water) in the mobile phase. Meanwhile, the Snyder-Soczewinski equation describes retention for adsorption processes:

$$(2) \qquad logk = logk_B - nlogX_B$$

where k_B is the retention factor with the pure stronger eluent B (water), n is the number of molecules of solvent B displaced by the analyte and X_B is the molar fraction of B in the mobile phase. Thus, a plot of log *k* versus φ_{water} should yield straight lines for systems where partition is the predominant retention mechanism while plots of log *k* versus log X_{water} should be linear for systems were adsorption is the predominant retention mechanism [68].

In a recent example, Hawkins *et al.* [78] studied the retention behaviour of nine N,N'-dialkylimidazolium-based ionic liquids on bare silica under HILIC conditions, by constructing plots of log *k* versus the linear and logarithmic functions of the water contents (referred to as V_B). They observed strong curvature in the log *k* vs. V_B plots, which was interpreted as clear evidence that partitioning was not the only mechanism contributing to the retention. In contrast, log *k* vs. log V_B plots showed a great linear fit, suggesting an adsorption process. Maksić *et al.* [79] observed linear plots ($R^2 \geq 0.9966$) when adjusting retention data of granisetron and two related substances on a zwitterionic phase to both retention models. Hence, they concluded that both adsorption and partition have great influence on the overall HILIC

mechanism of these analytes. In the study by Jovanović *et al.* [80] of the retention of iohexol and related substances on four different columns, they found that, for three of the phases, the retention data did not fit any of the models, while one of the phases based on bare silica showed linear plots for the adsorption model. They suggested that the formation of hydrogen bonds between the analytes and the silanol groups of the stationary phase may have been responsible for this behaviour.

The construction of ln k versus 1/T plots by recording retention data at different temperatures can also give additional information with respect to the retention mechanisms present, based on the Van't Hoff equation [72, 81]:

$$\ln k = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} + \ln \emptyset \qquad (3)$$

where ΔH° and ΔS° are the changes in enthalpy and entropy of the chemical process of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant (8.314 J/K.mol) and ø is the phase ratio V_S/V_m [82, 83]. The presence of curvatures in Van't Hoff plots are an indication of a shift in the predominant retention mechanism or the presence of multiple interactions. The calculation of the energies is also useful for determining whether retention of a particular analyte undergoes an exothermic or an endothermic process. For instance, Qiu *et al.* [84] evaluated the thermal behaviour of an in-house zwitterionic stationary phase constructing Van't Hoff plots of 15 compounds selected as model compounds. They observed that most of the solutes gave linear plots with positive slopes while some of the solutes showed curved plots with negative slopes. In other cases, some analytes were not influenced by changes in temperature, giving almost flat plots. Also in this work, an extensive discussion is given related to the thermodynamic data obtained. More recently, Takahashi *et al.* [85] conducted temperature studies on a triazole-bonded column of the derivatized L-tryptophan and the metabolite L-kynurenine. They observed linear plots with negative slopes that suggested the presence of a single type of interaction between the solutes and the triazole phase.

Another type of contributing interaction relevant to the HILIC mechanism is ion-exchange, as previously mentioned. McCalley proposed that plots of *k* as a function of the inverse of the M^+ concentration should exhibit linear relationships intercepting the origin if only ionic interactions are responsible for retention [71]. In this study, plots of *k vs.* $1/M^+$ showed curvatures that were attributed to the contribution of partition or adsorption mechanisms. Moreover, it was demonstrated that a group of basic analytes showed a contribution of ionic interactions of more than 50% for silica, amide and zwitterionic phases, but displayed little contribution in a diol phase. Another indicator of the presence of ionic interactions could be the influence observed on retention of changes in types of added salts or their concentration [71, 83, 86].

As mentioned, the properties of the stationary phase have a major influence on the retention mechanisms and, as such, the comparison of different columns has often been discussed in several studies. A description of the most important stationary phases in HILIC will be given in Section 1.1.2.1. later in this Introduction, highlighting their features and uses rather than the role of each one in the retention mechanisms.

One way of comparing different columns with different properties is to select a group of model compounds and evaluate differences in retention and selectivity. The retention studies described above can also give insights into differences in predominant interactions in different phases. Stationary phases have also been classified according to similarities in retention and the types of interactions that they promote, using chemometric methods such as principal component analysis (PCA). For example, Kawachi *et al.* [87] evaluated 15 stationary phases using retention data of a group of model compounds classifying them according to their hydrophilicity. Dinh *et al.* [88] evaluated the retention data of 21 model compounds on 22 columns finding linear distributions strongly related with the log D values of the analytes, suggesting that partition mechanism was the predominant retention mechanism for uncharged compounds. Evidence of the contribution of ionic interactions and hydrogen bonding was also observed.

The HILIC mechanism has also been studied using quantitative structure-retention relationship models, particularly the linear solvation energy relationship (LSER) model, which relates retention data with molecular structure descriptors. Each descriptor included in the model indicates the contributions of specific interactions to the overall retention. For instance, in the study by Chirita *et al.* [75], two additional descriptors (denoting the positive and negative charges of ionic solutes) to the five normally used were included to the equation of the conventional LSER model, finding significant contributions from the descriptors related to hydrogen bonding, partitioning and ionic interactions. Schuster and Lindner [89] used the same descriptors and observed contributions from hydrophilic partitioning for 68 solutes on 22 stationary phases, along with ionic interaction contributions for charged phases and solutes.

To support the hydrophilic partition mechanism, many authors [69, 90-93] have studied the formation of the water layer onto the surfaces of the HILIC stationary phases, illustrated in Figure 3. Data of the retention volume of benzene and toluene using high contents of ACN has been used to measure the pore volume occupied by the water layer immobilized on a silica surface. Their findings suggested that, at a water content range between 5% and 30% in the mobile phase, the water layer occupied from 4% to 13% of the pore volume of the stationary phase surface [90]. Using the same method, Greco *et al.* [91] demonstrated that the content of ACN and salt concentration influenced the thickness of the water layer adsorbed onto the stationary phase. In another study [92], high-field ^{2}H nuclear magnetic resonance (NMR) spectroscopy was used to identify the phase transitions of water present in bare silica and silica modified with sulfobetaine (zwitterionic) polymers, at and below freezing point. As expressed by the authors, water associated to polymer systems can be in three states being free (bulk water), freezable bound water (which has a slightly shifted phase transition temperature relative to bulk water) or nonfreezable water, which is defined as bound water that does not freeze in the normal temperature range expected for bulk water. As results, they observed the presence of nonfreezable water on the immediate surface of the stationary phases and that it was higher in

the sulfobetaine material compared to the silica one. Studies with materials of different pore sizes also suggested that the percentage of nonfreezable water decreased with the increasing of pore size. However, these experiments were conducted under aqueous conditions without the presence of organic solvents normally used in HILIC. More recently, coulometric Karl-Fischer titration has been used to study the uptake of water from the mobile phase for 12 different HILIC phases [94]. The authors of this study found that bare silica and monomeric bases such as amino and diol materials showed the formation of a monolayer of water on the surface followed by multilayer adsorption, while, for polymer grafted phases, the adsorption of water was through the swelling of the hydrogel layer. It was also proven that the size of the water layer is increased by the addition of higher concentrations of salts.

Many other studies [93, 95-97] have focused their efforts on broadening the knowledge related to the water layer formed onto HILIC phases. In summary, recent contributions indicate that the presence of hydrogen bonds plays an important role in water uptake, that the water layer increases as the percentage of water in the mobile phase increases and peaks between 20% to 40%, and that the water distribution from the pores of the stationary phase to the bulk solvent of the mobile phase is gradual until only organic solvent is present.

Indeed, a HILIC system is quite complex because the stationary phase can have particular properties, immobilize a water layer onto its surface and polar analytes are able to interact with this phase in several ways. Different strategies have been used to gain insights into HILIC retention mechanisms with the ultimate objective of predicting the retention times and selectivity of a particular group of analytes on a selected stationary phase. In the meantime, optimization of a HILIC method must be performed according to the columns available and taking into consideration the most important parameters influencing the separation.

1.1.2. Parameters affecting chromatographic separation

Both the characteristics of the stationary phase and the parameters of the mobile phase, such as organic solvent/aqueous proportion, pH and type and concentration of additive salts, highly affect the retention and selectivity of a HILIC separation. Furthermore, because in HILIC the stronger eluent is water, the injection solvent must have a high content of organic solvent to avoid distortion of the peak shape. All these parameters and its influence will be described in the following sections.

1.1.2.1. Stationary phases

As mentioned earlier, there are several stationary phases that can be used for HILIC purposes with different polarities and properties. In the comprehensive review by Hemström and Irgum [14], a full description is presented for each of the HILIC phases available to date, as well as their applications. Jandera [16] also reviewed the HILIC phases available including a section focused on monolithic columns. Very recently, Qiao *et al.* [98] reviewed the trends in stationary phase development over the last five years. Figure 4 depicts a summary of the most commonly used HILIC stationary phases.

Because the list of HILIC columns available is long and recent research is continuously adding more options, only the most commonly used phases are described below, as well as some considerations of the interactions that they can promote.

One of the first columns used for HILIC separations still frequently selected for several applications is the bare silica phase. The water layer formed onto this surface promotes the dissociation of free silanols, giving cation-exchange properties to the material that enhance the retention of basic compounds, while decreasing the retention of acidic analytes. Nowadays, there are different brands of silica phases specially designed for HILIC, such as Atlantis HILIC (Waters), Zorbax HILIC (Agilent), Alltima HILIC (Fisher Scientific), Kinetex HILIC (Phenomenex), Ascentis Express HILIC (Sigma-

Aldrich), etc., which might show some differences in selectivity attributable to changes in purity of the silica or the procedures used for column preparation [83].

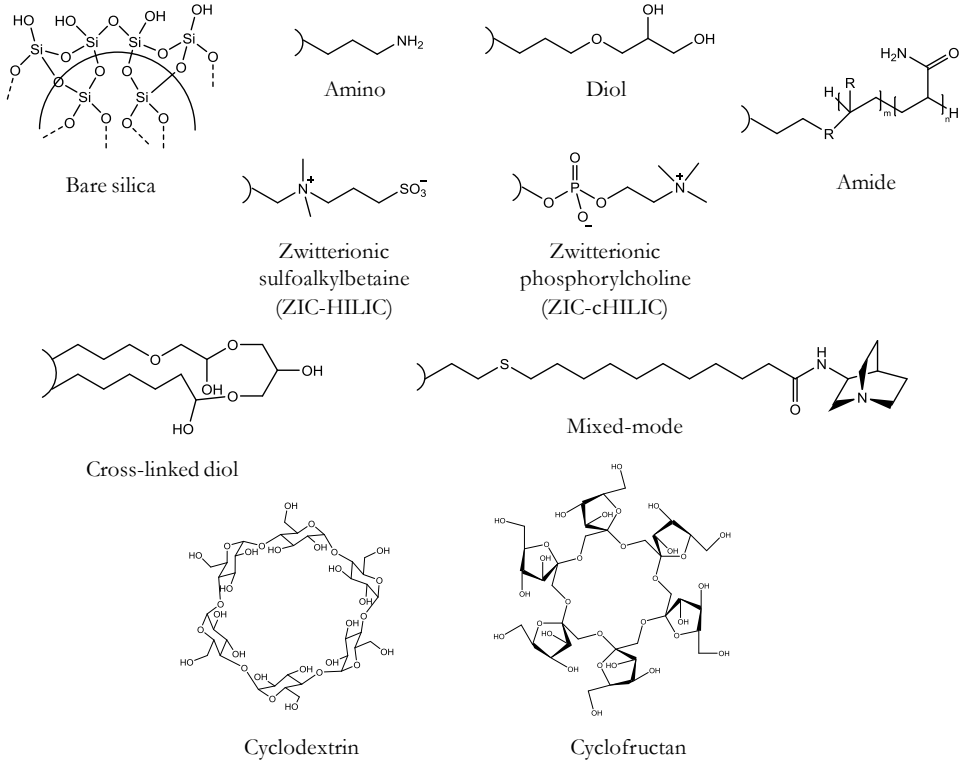

Figure 4. Structure of the most important HILIC phases.

Bonded silica phases with aminopropyl, diol or amide groups originally used for NPLC were used during the early years of HILIC. Some problems related to the limited stability of these phases in aqueous eluents that cause the release of the ligands were addressed in materials specially designed for HILIC. Because they have ligands with only one functional group, the interactions that they promote can be explained by the chemistry of these groups. Amine phases are the most polar of these phases and because amino groups can be protonated at certain pH levels, they can promote ion-exchange interactions with the analytes. However, due to the same

characteristics, they can react with some analytes, for example with carbonyl compounds in a Schiff's base formation reaction. Because the amide phase is not basic, it is less reactive, being more useful for several compounds and still suitable for a large number of applications. Phases with diol groups are similar to bare silica materials in terms of polarity. Besides, they contribute less to ion-exchange interactions and promote hydrogen bonding, which are properties desirable for HILIC. The Luna HILIC (Phenomenex) column, in which the diol groups are crosslinked through carbon chains, (Figure 4) has gained interest because it has adequate polarity for a broad range of compounds. Other silica-based materials like cyclodextrin-bonded phases have also been used, displaying interesting properties such as chiral recognition [23, 86]. These phases with bonded macromolecules such as sugars are still the subject of recent research [98, 99].

Columns based on polymeric resins are also used after modification with the desired functional groups. One of the most used HILIC phases is the zwitterionic column synthesized from sulfoalkylbetaine groups (Figure 4), which is available on silica or polymeric supports. ZIC-HILIC columns are well recognized in the manufacturing of these HILIC phases and, recently, the new phase ZIC-cHILIC was commercialized modified with phosphorylcholine groups (Figure 4). As previously mentioned, these types of phases have great capabilities for binding water onto their surface. The presence of quaternary amine groups and sulfonic acid groups in a 1:1 ratio in the same moiety supposes a zero net charge. Therefore, ion-exchange contributions are not highly significant. This phase can exhibit a low negative excess charge, probably because the sulfonic groups are located on the surface [14]. The phosphorylcholine phase has the charges in the opposite order, with the amine groups being the distal ones. Recent research on column development has focused on in-house zwitterionic materials after the success of the ZIC-HILIC columns. Different ionic functional groups, such as carboxylic, imidazolium, tertiary, secondary or primary amine groups, have been introduced to silica or polymeric particles and polymeric monoliths to give zwitterionic properties to the stationary phases. These groups were either included or not in the same ligand attached to the substrates and the

charges organized in different spatial arrangements. Figure 5 illustrates different spatial distributions of the charges for different zwitterionic phases. Several of these phases have shown comparable or better results than commercial phases exhibiting great chromatographic features. A summary of these novel zwitterionic phases can be found in a recent review [98].

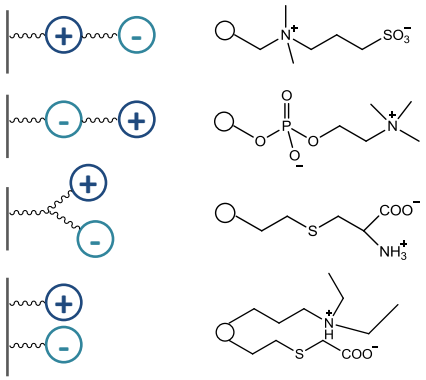

Figure 5. Different spatial arrangements of zwitterionic phases.

With respect to the HILIC phases available, several of them have mixed-mode properties that can exhibit reversed-phase, HILIC or ion-exchange properties together in the same material. Because they are capable of providing flexible selectivity for a large number of compounds, recent progress has focused on the development of materials with these characteristics. In addition, HILIC monolithic columns are gaining interest due to the physical and mechanical advantages that they can offer, such as higher column permeability and faster separations because they can withstand higher flow-rates [16, 98].

1.1.2.2. Mobile phase

After the selection of the stationary phase, the optimization of the mobile phase parameters such as organic solvent/water proportion, pH and additives must be performed. The mobile phase in HILIC is less hydrophilic than the stationary phase, in contrast to RPLC separations. In theory, the organic solvent can be selected from a list of polar protic (e.g. MeOH,

ethanol, isopropanol) or polar aprotic (e.g. ACN, tetrahydrofuran) solvents. Polar protic solvents are both donor and acceptor of hydrogen bonds, while polar aprotic solvents can only be acceptors. As such, protic eluents tend to compete with water for polar sites on the surface of the stationary phase, affecting the water layer formed on the phase surface [82]. For this reason, the use of eluents like MeOH often implies less retention of the polar compounds. Normally, ACN is selected as an organic solvent because it provides greater retention and better shaped chromatographic peaks [16, 82]. The mobile phase usually contains 5% to 40% of water or buffered aqueous phase containing volatile salts (if the LC system is intended to be coupled to MS-based detectors). Preliminary experiments must be performed at high contents of water to ensure the complete elution of the analytes and, starting from that point, elution strength can be adjusted, increasing the percentage of ACN. A gradient profile can be used for difficult separations starting from ~5% of water up to ~40%, while decreasing the amount of ACN [16]. A minimum percent of water is needed to ensure the formation of the water layer on the surface of the stationary phase.

In HILIC, mobile phases are frequently buffered and their pH and ionic strength can affect retention and separation. Common buffered aqueous mobile phases include $HCOONH_4$/HCOOH and CH_3COONH_4/ CH_3COOH solutions. The effect of a buffered mobile phase is of special interest when the stationary phase or the analytes have ionizable groups, as ion-exchange interactions can be shifted by controlling the pH. Charged compounds are more hydrophilic than their neutral form and, therefore, they are more retained in HILIC [83]. In addition, as mentioned above, changes in the charge state of the stationary phase contribute with repulsive or attractive interactions to retention. Bare silica and silica-based neutral phases exhibit ionized silanol groups at pH values above 5 that enhance retention of basic compounds but decrease retention of acidic analytes. Sometimes though, differences between low and high pH values are not as significant as in RPLC because the high content of organic solvents limits the ionization [16]. It has been discussed whether aqueous pH data ($^{W}_{W}pH$) should be used instead of the pH measured in the aqueous/organic solution ($^{S}_{W}pH$), as there are

differences when a high content of organic eluent is used. The most popular choice is to use $^{w}_{w}pH$ values because it is considered more appropriate, as retention is promoted by a water layer immobilized on the stationary phase, and also pK_a values are normally referred to aqueous solutions [68, 83].

Neutral compounds also undergo differences in retention when varying buffer concentrations, which is attributable to an increase in the thickness of the water layer. At higher salt concentrations, the solvation of the salt ions increases the size of the water layer or creates a salting-out effect by making the analytes more accessible to the stationary phase. However, for stationary phases with ionic functionalities, the addition of salt might decrease HILIC retention as the counter-ions of the salts may compete with the analytes for the ionic groups of the stationary phase [16]. The selection of the type of salt is very important as they might involve different elution strengths [76]. It has been observed that the presence of salt additives in the mobile phase reduces electrostatic interactions between ionized compounds and the stationary phases [100]. When electrostatic attraction is present, an increase in the salt concentration often leads to reduced retention of the charged solutes, whereas, in repulsive interactions, the increase of salt concentration results in increased retention [76, 83, 101, 102]. Salt concentration can be referred to the aqueous phase of the mobile phase or the overall mobile phase. In some cases, the concentration of salt is maintained constant throughout gradient profiles by adding the salt to both the organic and the aqueous phase to maintain ion-exchange contribution that could affect separation or peak shape [71].

Observations of the impact that the different chromatographic parameters described above can have on separation and retention are very helpful when optimizing a HILIC method. However, because retention mechanisms are quite complex and there are so many options available with respect to stationary phases and mobile phase, their influence must always be assessed experimentally for any particular separation if previous results are not available. For this purpose, either univariate or multivariate analysis can be used. Frequently, the univariate approach is preferred by testing one variable

at a time, while keeping other parameters constant. However, interactions between parameters are not considered when using this approach, so an experimental design might give insights on this matter. The main drawback of multivariate approaches is that relatively time-consuming data handling is needed and the total number of experiment trials cannot be less than the univariate strategy. In any case, an exhaustive discussion of the optimized parameters and the levels tested as well as the conclusions made in each step is often missing in several studies of HILIC applications, in spite of being very important for researchers not familiarized to this technique. For this reason, a detailed description of the optimization step is very helpful when a particular group of analytes are being separated by HILIC for the first time or new applications in this chromatographic mode are proposed [103].

1.1.3. HILIC applications

The number of HILIC studies has grown substantially in recent years, being applied to several groups of polar compounds relevant to environmental, pharmaceutical, biological and other fields. Several publications reviewing important HILIC applications are available in the literature focusing on a specific group of analytes (e.g. peptides [104, 105], antibiotics [106]) or types of stationary phases (e.g. [99, 107]). A book edited in 2011 by Wang and He was published by CRC Press, entitled Hydrophilic Interaction Liquid Chromatography (HILIC) and Advanced Applications, which includes 23 chapters discussing the most advanced HILIC applications in the fields of environmental sciences, food analysis, clinical chemistry, pharmaceutical research and biotechnology discovery, written by well-known contributing authors [108].

Recent HILIC-MS applications in the environmental field have been compiled in a review paper to be submitted in the journal *Trends in Analytical Chemistry*. In this publication, included in the following section, a review of the latest HILIC methods developed for environmental applications is performed focusing on the stationary phases and chromatographic conditions frequently used, as well as the advantages given by the use of this LC mode for the specific analytes and matrices.

1.1.3.1. Hydrophilic interaction liquid chromatography coupled to mass spectrometry-based detection to determine emerging organic contaminants in environmental samples

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY-BASED DETECTION TO DETERMINE EMERGING ORGANIC CONTAMINANTS IN ENVIRONMENTAL SAMPLES

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Abstract

Hydrophilic interaction liquid chromatography (HILIC) has gained well acceptance as an alternative to reversed-phase liquid chromatography (RPLC) for the retention and separation of polar compounds. Because a great number of the emerging organic contaminants are polar, this supposes a progress to solve most of the problems and limitations found in the conventional methods developed for their determination. In this review, recent HILIC applications developed in the environmental field are described which are normally coupled with mass spectrometry, in order to reach the required limits to quantify contaminants present at trace levels and benefit from its confirmation power. Special attention is paid to the stationary and mobile phases commonly used in terms of the advantages that offer compared with reversed-phase columns and the high water content mobile phases traditionally used in RPLC. The most important features related to the matrices and contaminants normally studied are also discussed together with the contribution of the HILIC mode to reduce matrix effect.

Keywords: *hydrophilic interaction liquid chromatography; mass spectrometry; emerging organic contaminants; environmental samples.*

1 Introduction

Hydrophilic interaction liquid chromatography (HILIC) is a relatively new chromatographic mode developed in response to the lack of retention of polar compounds in reversed-phase liquid chromatography (RPLC) [1]. When using liquid chromatography (LC), RPLC is the preferred mode and the C_{18} columns are the stationary phases most commonly used, thanks to their versatility and robustness. However, sometimes the separation of polar compounds is difficult to achieve and they also tend to elute close to the void volume, even when a high content of water in the mobile phase is used. This is a significant drawback when coupling LC to mass spectrometry (MS)-based detection, because high contents of water in the flow that enters the instrument difficult ionization and, in addition, the salts and polar interferences that co-elute with the analytes at the beginning of the chromatogram can cause suppression or enhancement of the response, known as matrix effect (ME) [2,3]. To overcome these problems, Alpert [4] introduced the term HILIC to describe a chromatographic mode based on the combination of a polar stationary phase with a mobile phase containing water and an organic solvent, where the aqueous phase was the stronger eluting solvent.

In his study, Alpert proposed that the mechanism of HILIC consisted in partitioning between a water-enriched layer partially immobilized on the stationary phase and the mobile phase being mainly polar organic solvent. Since then, a large part of the research dedicated to HILIC has focused on its mechanism [2,5-7]. Recently, Guo [8] reviewed the progress made so far to understand HILIC mechanism, focusing on fundamental aspects such as the water layer absorbed onto the stationary phase, the selectivity of several stationary phases and the kinetic performance of the technique. Frequently, the predominant mechanism of HILIC is hydrophilic partitioning (as mentioned before) and the presence of the water-enriched layer onto the surface of the stationary phases has been experimentally demonstrated. However, other mechanisms like surface adsorption or electrostatic interactions also play an important role in the retention, which gain relevance depending on several factors such as the organic solvent content. In general, retention in HILIC is very complex and

depends on the type of stationary phase, mobile phase composition and the properties of the analytes.

In fact, as several interactions can contribute to retention in HILIC, the selection of the stationary phase might not be as straightforward as in RPLC, where partition is the main interaction and retention can be more or less predicted on phases like C_{18} and C_8 according to the polarity of the compounds. Each of the stationary phases available for HILIC promotes specific interactions which can deliver different retention behaviour and selectivity for a specific group of compounds. During the last decade, several authors have reviewed the different HILIC stationary phases used since the introduction of this technique, focusing on the properties and applications of bare silica, bonded-silica with polar groups such as amide, diol, zwitterionic, and macromolecules, ionic-exchangers, mixed-mode phases and others [6, 9-11]. Recently, Qiao et al. [12] discussed the updates and recent progress made in the development and characterization of HILIC stationary phases, mostly focusing on zwitterionic, mixed-mode, monolithic and macromolecules-bonded phases.

The mobile phase conditions such as the organic solvent, pH and salt concentration also have great influence on the retention of the analytes [7]. Frequently, the preferred organic solvent is acetonitrile (ACN) and the aqueous phase contains additives like salts and acids, which are used to control the pH and ionic strength during the analysis, and should be volatile to avoid problems in the interface with MS-based detectors [11]. The fundamental aspects, influence and trends of the parameters affecting the mobile phase to be considered when optimizing a method in HILIC have been thoroughly discussed [7, 13, 14].

HILIC was early developed for the separation of carbohydrates, peptides, nucleic acids or proteins, but since then, this chromatographic mode has been applied to determine several polar and hydrophilic compounds in different matrices such as biological samples, foodstuff or environmental samples, contributing to the fields of metabolomics, proteomics, pharmaceutical, environmental and the food industry [7, 15]. A large number of emerging organic contaminants (EOCs) found in environmental samples has polar properties, because their solubility

in water facilitates their transport trough wastewaters into the environment. In 2011, van Nuijs et al. [1] reviewed several methods based on HILIC applied to food and environmental samples. The methods related to the environmental field focused on pharmaceuticals (estrogens, cytostatic drugs, antibiotics, metformin, contrast agents, etc.), drugs of abuse and pesticides mainly in river, surface, drinking and wastewater samples. Also in 2011, Li *et al.* [16] reviewed the HILIC methods available to determine several contaminants in environmental samples, giving special attention to the sample preparation. Since then, new methods with environmental applications have been developed which have incorporated recent advances on the technique.

The aim of the present review is to discuss recent applications of HILIC to environmental matrices, paying special attention to the advantages and contribution that this chromatographic mode has offered to the environmental field.

2 Stationary phases

There are several phases that can be used for HILIC applications which are able to promote different interactions such as hydrophilic partition, cation or anion-exchange or hydrogen bonding, yielding different retention behaviour, elution order and selectivity. In RPLC, the C_{18} stationary phase is often suitable for a wide range of compounds for which is considered quite versatile whereas in HILIC there is not such universal column [6]. Manufacturers have developed phases with different chemistry to promote particular mechanisms, ranging from materials that can behave as RP or HILIC phases (depending on the mobile phase) to columns with ionic functionalities. As previously stated, the properties of the different HILIC stationary phases and their applications have been extensively reviewed [6, 9-12]. In this section, the stationary phases most frequently used for environmental applications will be discussed, as well as relevant observations made related to their performance.

The HILIC stationary phases commonly used in the environmental field are the bare silica, zwitterionic, amide and diol

phases, as showed in Table 1. The most frequently used brands of columns packed with unmodified bare silica gel are the Atlantis HILIC from Waters and the Kinetex HILIC from Phenomenex. Columns with zwitterionic functionalities are silica or polymer-based and frequently bonded with sulfoalkylbetaine moieties that contain sulfonic acid and quaternary amine groups separated by a short alkyl chain [9].

Table 1. Stationary phases, mobile phases and type of elution used in environmental applications under HILIC conditions.

Stationary phase	Column	Mobile phase	Elution	Ref.
Bare silica	Ascentis Express HILIC	A: ACN, B: 15 mM CH_3COONH_4/CH_3COOH aqueous buffer (pH 4.5)	gradient	[48]
	Atlantis HILIC	A: ACN, B: H_2O (both 0.1% HCOOH)	gradient	[43]
	Altima HP	A: ACN, B: H_2O (both 0.1% HCOOH)	isocratic	[27]
	ZORBAX Rx-SIL HILIC	A: ACN, B: 2 mM CH_3COONH_4/CH_3COOH aqueous buffer (pH 4.5)	gradient	[20, 54, 57]
	Atlantis HILIC	A: ACN, B: isopropanol, C: 200 mM $HCOONH_4/HCOOH$ aqueous buffer (pH 3)	isocratic	[42]
	Atlantis HILIC	A: ACN, B: aqueous 20 mM $HCOONH_4$	gradient	[52]
	Kinetex HILIC	15:10:75 $HCOONH_4$ (pH 3.5):MeOH:ACN	isocratic	[23]
	Atlantis HILIC	A: ACN, B:H_2O	gradient	[55]
	Kinetex HILIC	A: ACN, B: H_2O (both 0.1% HCOOH and 2 mM $HCOONH_4$)	gradient	[44]

Table 1. (Cont.)

Stationary phase	Column	Mobile phase	Elution	Ref.
ZIC-HILIC	ZIC-HILIC	A: ACN, B: aqueous 60 mM HCOOH	isocratic	[50]
	ZIC-HILIC	A: ACN, B: H_2O (both 0.1% HCOOH)	Gradient	[53]
	Nucleodur HILIC	A: ACN, B: MeOH, C: 20 mM CH_3COONH_4/CH_3COOH aqueous buffer	pseudo isocratic	[41]
	ZIC-HILIC	A: ACN, B: 10 mM $HCOONH_4$/HCOOH aqueous buffer (pH 3)	gradient	[24]
	ZIC-HILIC	A: ACN, B: 2:3 aqueous 30 mM CH_3COONH_4:ACN (pH not adjusted)	gradient	[56]
	ZIC-HILIC	A: ACN, B: H_2O (both 5% CH_3COONH_4 and 0.01% HCOOH)	gradient	[45]
	Luna C18 and ZIC-HILIC (in series)	A: 5% aqueous 5 mM CH_3COONH_4 (pH 6.8) in ACN, B: 25% aqueous 5 mM CH_3COONH_4 (pH 6.8) in ACN	gradient	[32]
	Syncronis	A: ACN, B: 100 mM $HCOONH_4$/HCOOH aqueous buffer (pH 3.75)	gradient	[22]
	ZIC-HILIC	A: ACN, B: 2 mM $HCOONH_4$/HCOOH aqueous buffer (pH 3)	gradient	[29]
	Nucleoshell HILIC	A: 95:5 ACN:2 mM CH_3COONH_4, B: 97:3 2 mM CH_3COONH_4:ACN (both 0.05 % HCOOH)	gradient	[33]

Table 1. (Cont.).

Stationary phase	Column	Mobile phase	Elution	Ref.
Zwitterionic	Poroshell 120 EC-C18 and ZIC-HILIC (in series)	A: ACN, B: H_2O, C: RPLC mobile phase (aqueous 10 mM CH_3COONH_4/ACN)	gradient	[31]
	ZIC-HILIC	A: ACN, B: H_2O (both 0.1% HCOOH)	gradient	[47]
	ZIC-HILIC	A: ACN, B: H_2O (both 0.1% HCOOH)	gradient	[58]
	ZIC-cHILIC	ACN/CH_3COONH_4/CH_3COOH aqueous buffer (pH 5)	isocratic	[17]
	ZIC-HILIC	A: 5% aqueous 20 mM $HCOONH_4$ (pH 4) in n-propanol, B: 50% aqueous 20 mM $HCOONH_4$ (pH 4) in n-propanol, C: 100 mM aqueous $HCOONH_4$ (pH 4)	gradient	[59]
Amide	TSK-gel Amide-80	A: ACN, B: aqueous 0.05% TFA	isocratic	[50]
	XBridge amide	A: ACN, B: H_2O	isocratic	[28]
	TSK-gel Amide-80	A: 5% solvent B in ACN, B: 4mM $HCOONH_4$ (both pH 3.5 using HCOOH)	isocratic	[39]
	TSK-gel Amide-80	A: ACN, B: H_2O (both 50 mM HCOOH)	gradient	[46]
	TSK-gel Amide-80	A: 5% H_2O in ACN, B: H_2O (both 5 mM $HCOONH_4$ and 3.6 HCOOH, pH 3.5)	gradient	[34]
	Acquity UPLC BEH Amide	A: 5% H_2O in ACN, B: H_2O (both 1 mM $HCOONH_4$ and 0.01% HCOOH)	gradient	[38]

Table 1. (Cont.).

Stationary phase	Column	Mobile phase	Elution	Ref.
Diol	Luna HILIC	A: 5% H_2O in ACN, B: H_2O (both adding 5 mM CH_3COONH_4 at pH 3.5)	gradient profile	[40]
	Luna HILIC	A: ACN, B: aqueous 10 mM CH_3COONH_4	gradient profile	[49]
	Luna HILIC	A: ACN, B: aqueous 5 mM CH_3COONH_4	gradient profile	[19]
	Luna HILIC	A: ACN:MeOH 87.5:12.5, B: aqueous 5 mM CH_3COONH_5	gradient profile	[60]
Other	Restek Ultra IBD phase (polar-embedded alkyl)	A: 0.1 % HCOOH in ACN, B: aqueous 10 mM $HCOONH_4$ (2.9)	gradient profile	[35]
	Restek Viva PFPP	A: ACN, B: H_2O (both 0.1% HCOOH)	gradient profile	[18]
	ZORBAX SB-C18 and Venusil HILIC (in series)	A: ACN, B: aqueous 10 mM CH_3COONH_4 (both 0.1% HCOOH)	gradient profile	[30]

There are several columns with zwitterionic properties commercially available, but as it can be noted in Table 1, the most used one is the ZIC-HILIC phase manufactured by Merck. This commercial house also developed the ZIC-cHILIC phase with phosphorylcholine moieties having phosphoric acid groups instead of sulfonic acid groups and interchanging the order of the charges in the ligand. Because this column is relatively new, environmental applications using it are less common [17]. Other zwitterionic columns with similar functionalities which are less

frequently used but commercially available are Nucleodur, Syncronis or Nucleoshell. Amide and cross-linked diol phases are also frequently used being the TSKgel Amide-80 (Tosoh Bioscience) and the Luna HILIC (Phenomenex) the most used ones.

Several HILIC studies discuss the comparison among different polar stationary phases, to identify the most suitable one for a group of analytes [18, 19]. In the study of van Nuijs *et al.* [19], a Luna HILIC (cross-linked diol) column was selected for the separation of 9 drugs of abuse because it provided better sensitivity and robustness when compared with a Zorbax RX-Sil silica phase, obtaining good retention and satisfactory separation within 8 minutes of analysis in the LC-triple quadrupole (QqQ) system. The authors compared their results with a previous study where the Zorbax RX-Sil silica column showed late elution for ecgonine methyl ester causing higher matrix effect in the ion trap MS detector, as ionic interferences also eluting last can affect the ionization [20]. Therefore, the limit of quantification (LOQ) for this compound was improved in the method using the cross-linked diol phase. Bisceglia *et al.* [18] published an interesting study where 13 stationary phases were compared for the separation of 23 drugs of abuse including three HILIC phases, RP phases with embedded polar functionalities and even phases that can be operated in both RPLC and HILIC modes. They observed enhanced retention of the compounds on the HILIC phases Luna HILIC (cross-linked diol), Obelisc-N (zwitterionic) and Ultra IBD (embedded polar group, proprietary), especially for those highly polar such as ecgonine and anhydroecgonine. However, resolution was poor, concluding that HILIC might be an alternative for RPLC when retention problems cannot be addressed, but might not be suitable for the separation of a large number of analytes.

Another interesting comparison can be drawn from three methods describing the separation of artificial sweeteners using three different stationary phases: zwitterionic, bare silica and cross-linked diol [21-23]. Even when the mobile phase conditions and flow-rate used were different in each case, as shown in Table 2, some differences were evident like the higher retention obtained for the

Table 2. Retention times obtained in three different methods describing the separation of a group of artificial sweeteners in three different stationary phases: zwitterionic [22], bare silica [23] and cross-linked diol [21].

Stationary phase	**t_R artificial sweeteners**					
Zwitterionic	ACE	SAC	CYC	SUC	NHDC	ASP
(ACN/100 mM $HCOONH_4$/HCOOH; 0.5 mL/min)	1.2	1.6	4.2	5.5	7.4	7.9
Bare silica	ACE	SAC	CYC	SUC	NHDC	ASP
(ACN/MeOH/5 mM $HCOONH_4$/HCOOH, 0.1 mL/min)	2.0	2.0	2.1	2.6	2.7	5.2
Cross-linked diol	ACE	SAC	SUC	CYC	NHDC	ASP
(ACN/5 mM CH_3COONH_4, 0.2 mL/min)	1.8	1.9	2.2	2.3	4.0	5.5

zwitterionic stationary phase and differences in selectivity. Interestingly, the elution order was the same in all cases. Figure 1 compares the separation obtained for the bare silica and zwitterionic stationary phases during the optimization step of one of the studies above [22], showing how the zwitterionic phase gave better separation.

The use of polar stationary phases in the HILIC mode has had significant advantages in the separation of several pollutants. For instance, Scheurer *et al.* [24] used a zwitterionic phase (ZIC-HILIC) for the separation of the antidiabetic pharmaceutical metformin which shows almost no retention in RPLC phases, due to the strong basic properties of this compound. Using this LC mode, they reported the first results on metmorfin occurrence in environmental waters in Germany. Apart from poor retention on RP stationary phases, basic compounds can also exhibit peak tailing on silica based RP phases due to the additional retention that negatively charged residual silanols provide trough ion-exchange interactions [25]. In this sense, HILIC has contributed to solve the problems associated to this type of compounds [26]. For instance, the determination of the antibiotics spectinomycin and lin-

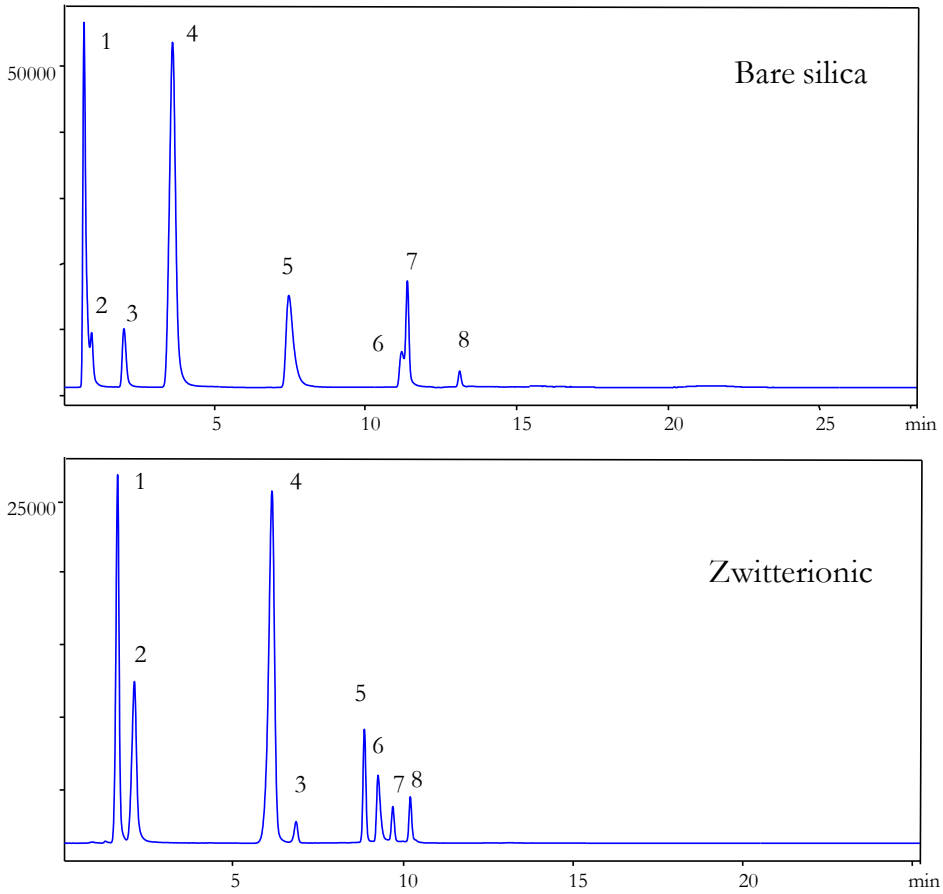

Figure 1. Chromatographic separation of a group of artificial sweeterners on bare silica and zwitterionic stationary phases obtained during the HILIC optimization step [22]. Peak identities: (1) acesulfame, (2) saccharine, (3) sucralose, (4) cyclamate, (5) neohesperidine, (6) aspartame, (7) stevioside, (8) glycyrrhizic acid.

comycin in environmental samples was improved in terms of retention, separation and peak shape using a bare silica stationary phase under HILIC conditions [27].

Several of the studies using HILIC for environmental applications have compared the HILIC methods with RPLC methods [18-21, 27-29]. For example, Chao *et al.* [28] compared the performance of the RP column XBridge C_{18} and the HILIC phase XBridge Amide to separate fullerols (hydroxylated

fullerenes), finding poor retention in the C_{18} phase when compared with the amide phase. They attributed this behaviour to the incapability of the hydrophobic core of the analytes to interact with the RP phase due to the presence of the hydroxyl groups on the surface of the fullerols. Instead, the amide phase exhibited efficient retention of the analytes using 90% ACN in the mobile phase. In another study [29], three RP stationary phases (Ascentis Express C_{18}, Zorbax Eclipse XDB-C_{18} and the Ascentis Express RP-amide with polar groups embedded) were compared with the ZIC-HILIC column for the separation of five iodinated X-ray contrast media, being highly polar compounds. In their comparison, the authors found poor retention of the analytes in the RP phases requiring high water content in the mobile phase in order to provide some retention, which highly affected the ionization in the LC-MS instrument. In contrast, the HILIC method offered higher retention and enhanced MS response; however, the separation of isomers that was observed in some of the RP phases was not possible in the HILIC column. Ordoñez *et al.* [21] also compared RPLC and HILIC methods for a group of artificial sweeteners using the Luna C_{18} and Luna HILIC (diol) columns. They found better LOQs for the HILIC method but higher matrix effect and lower reproducibility when compared to the RPLC separation. For this reason, they preferred the RPLC method over the HILIC one.

Because they exhibit alternative selectivity, the use of RPLC and HILIC in a single method is frequent, either coupling columns in series [30-32], injecting the sample in two different methods (HILIC and RPLC) [33-35], or using comprehensive two-dimensional chromatography [36, 37]. However, to the best of our knowledge there are no recent environmental applications using the latter strategy. The approach of injecting the sample separately in HILIC and RPLC methods has been widely used in recent years, thanks to the additional information that provides by separating peaks that are not resolved by using only one LC mode. For example, Lajeunesse *et al.* [34] have performed the chromatographic separation of a list of cyanotoxins in two groups, the polar ones on an amide phase while the less polar ones on a C_{18} phase, solving problems of low retention and distortion of peak shapes. Bisceglia et al. [35] also

used a reversed phase (pentafluorophenyl-propyl) for the separation of cocaine and cocaine metabolites, and a polar-embedded alkyl phase under HILIC conditions to specifically analyze ecgonine and anhydroecgonine, because these two analytes showed no retention under RP conditions. Very recently, Christophoridis *et al.* [38] used a HILIC phase (amide) and a RPLC phase (C_{18}) to separate isomeric transformation products (TPs) obtained during the ozonation of ranitidine, which co-eluted when using only the RPLC method. Injecting the sample in two different chromatographic methods could double the analysis time, whereas the coupling of columns might deliver a faster analysis depending on the conditions used.

The coupling of RPLC and HILIC stationary phases in series benefits from the raising percentage or organic solvent in the usual gradient of RPLC, because HILIC separations start with a high proportion of organic solvent. Chen *et al.* [30] and Rajab *et al.* [31] reported methods using serial coupling to determine toxins [30] and TPs of diclofenac [31] in environmental samples. In the first case, the separation of 19 toxins with lipophilic and hydrophilic properties was achieved on a ZORBAX SB-C_{18} column coupled to a Venusil HILIC (amide) column. The authors observed how the C_{18} column retained the 8 hydrophobic toxins but did not retain the 11 toxins with hydrophilic properties, which were retained and resolved in the HILIC column. Likely, in the HILIC phase the hydrophobic toxins eluted at void volume, except for yessotoxin, which eluted the last due to its enhanced retention in both columns [30]. In the other study, the diclofenac TPs obtained after oxidation with a boron-doped diamond electrode were separated in a single injection coupling Poroshell 120 EC-C_{18} and ZIC-HILIC columns, offering a more efficient, faster and easier approach to study TPs in complex samples [31].

3 Mobile phases

The selection of the mobile phase conditions in HILIC separations is very important to obtain satisfactory results, so a section discussing their optimization is often included in several publications. At the beginning of the analysis, HILIC separations are performed at high content of organic solvent in opposition to RPLC where high contents of water are needed to retain polar analytes. This supposes an

advantage when using MS-based detection as ionization and desolvation processes in the ion sources are favored when using high proportions of organic solvent. In addition, the use of salts and buffer solutions is more common in HILIC, as it is more sensitive to pH changes and the presence of salts can influence retention and selectivity [6, 7]. This behavior is related with the different retention mechanisms involved in HILIC and the influence of mobile phase parameters on the water layer immobilized on the polar stationary phase. These effects are more evident when ionic interactions are present, as the analytes can change their charge state from charged to neutral improving or decreasing retention. It has also been proved that an increase in salt concentration increases the thickness of the immobilized water layer, which delivers higher retention if hydrophilic partitioning is predominant in the overall mechanism [5, 8].

Table 1 shows how the organic solvent commonly used in environmental applications is ACN, as is common for HILIC approaches in general. In addition, the mobile phases usually contained ammonium acetate or ammonium formate in concentrations up to 200 mM, and were normally adjusted to acidic pH values between 3 and 4.5 using formic acid (preferred acid). This buffered solutions allowed to control pH and ionic strength during separation. These additives were placed either in the aqueous phase or in both the organic and the aqueous phase. For example, Halme *et al.* [39] tested different organic/aqueous proportions, three pH values (3, 3.5 and 4) and three salt concentrations (2, 4 and 10 mM) for a mobile phase containing ACN and an aqueous $HCOONH_4$/HCOOH buffer solution to separate a group of toxins. The final conditions were 4 mM of the buffered aqueous phase and ACN (with 5% of the aqueous phase), both adjusted at pH 3.5 using HCOOH at 40:60 under isocratic mode, because it gave better peak shape, resolution and a faster analysis. Ordóñez *et al.* [40] optimized the HILIC mobile phase for the separation of artificial sweeteners on a Luna HILIC (cross-linked diol) column by testing different salt additives (CH_3COONH_4 or $HCOONH_4$) at 5 mM adjusted either with CH_3COOH or HCOOH at different pH values from 3.5 to 6.5. In this study, the retention of the analytes increased as pH was raised but one of the analytes

(aspartame) showed peak broadening. For this reason, and because $HCOONH_4$ caused peak splitting of acesulfame, the optimum salt was CH_3COONH_4 and solution was adjusted at pH 3.5.

The addition of a small percentage of MeOH to the mobile phase can improve the sensitivity in some cases, as demonstrated in a method for the determination of 19 acidic herbicides and metabolites in river water samples where the response of some analytes was increased by a factor of 2 to 10 [41]. This fact was attributed to a better ionization in the ESI source due to the protic character of MeOH. In this study, different salt concentrations (20 to 200 mM) in a CH_3COONH_4 buffered aqueous phase were tested observing that this increase in ionic strength did not suppose any substantial change in retention, thus 20 mM was selected. The authors also obtained lower instrumental detection limits (LOD) when compared with RPLC methods, demonstrating the advantages of HILIC with regard to an enhanced ionization. The high amount of organic modifier contributes to the formation of smaller droplets in the ion source, facilitating ionization and desolvation [3]. Similar to the previous study, Hayama *et al.* [42] also observed an increase in sensitivity when adding isopropanol to the ACN/buffered aqueous $HCOONH_4$/HCOOH (pH 3) mobile phase.

Salts or acids were sometimes added in both the organic and aqueous phases, especially when gradient elution was used, in order to maintain the ionic strength and pH of the mobile phase constant during the entire analysis [33, 43-46]. This fact can be observed in Table 1 where several methods add the same percentage of HCOOH (for example 0.1 %) to both aqueous and organic phases or the same concentration of salt. It is also noticeable how the gradient elution mode was preferred over isocratic elution. In HILIC, the use of isocratic elution is sometimes preferred to avoid the long equilibration times that are frequently needed when using gradient profiles [39]. Nevertheless, the use of this elution mode is often not possible if separation is not satisfactory or if complete elution requires even longer times than column stabilization.

As mobile phase in HILIC contains high proportions of ACN and the aqueous phase is the strong eluting solvent, the

injection solvent in HILIC must also contain high proportions of organic solvent or be 100 % organic solvent if possible, in order to avoid any distortion of the peak shape. For this reason, the direct injection of extracts from the sample preparation steps is possible when using HILIC separations. In a previous study [22], we reported the direct injection in the LC-high-resolution MS (HRMS) system of the SPE extracts in the NH_4OH :MeOH:ACN (1:4:15) solution used for the elution of the analytes in the SPE procedure, avoiding evaporation steps, which allowed the simplification of the procedure while achieving similar LOQs. Barbaro *et al.* [47] also injected the methanolic eluting fraction obtained from the SPE performed in a Oasis HLB cartridge directly into the HILIC column. Halme *et al.* [39] treated freeze-dried algae samples using HILIC mobile phase (40:60, 4mM ammonium formate buffered aqueous phase adjusted at pH 3.5: 5% buffered aqueous phase in ACN) as extracting solvent which was directly injected in the LC instrument after spiking with internal standards. The development of an on-line SPE-HILIC method was also possible thanks to the compatibility of HILIC with organic solvents by directly transferring the methanolic fraction eluted from the SPE to the column [48]. The first HILIC method using large-volume injection (LVI) injected 750 μL of the samples containing the analyte (acrylamide) using dichloromethane as injection solvent, as it proved to better focus the analyte in the head of the column in comparison with acetone and ethyl acetate, as can be seen in Figure 2 [43]. Even when several other methods have directly injected organic extracts obtained from preparation procedures [30, 42, 45, 49], the change of solvents by evaporating and reconstituting the sample is more common, probably because optimized extracting solvents are incompatible with HILIC or to preconcentrate the sample. In order to fully benefit from all the advantages of this LC mode, the injection of organic extracts is a great option to simplify the method and reduce manipulation of the sample.

4 Application to environmental samples

Within the environmental field, HILIC has been mainly applied to environmental water samples including drinking water, tap water, surface water such as river,

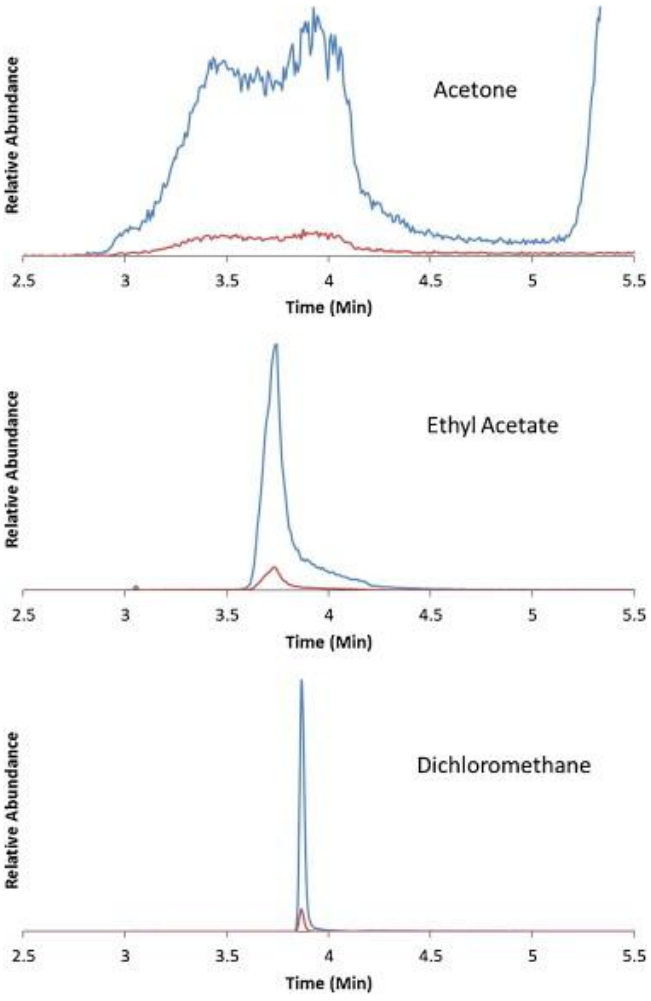

Figure 2. The effect of three different sample solvents (acetone, ethyl acetate, anddichlormethane) on the peak shape of acrylamide when injecting volumes of 750 μL [43].

creek and lagoon waters, irrigation water and wastewaters from urban treatment plants or hospitals, as it can be noted from Table 3. Among these samples, urban wastewater and river water samples are the most studied ones, as they give information of the consumption and transport of the EOCs to the sewages, their transformation during the treatment processes and their release into the environment [20, 22-24, 29, 35, 48]. Other types of

environmental matrices analyzed using HILIC include algae [34, 39], cyanobacteria [46, 50, 51] and in a lesser extend aquatic organisms [44], fish tissue [52], soil [45] and manure [27].

Table 3 shows how the type of EOCs that have been more frequently determined using HILIC are pharmaceuticals, toxins and drugs of abuse. Among pharmaceuticals, the list of analytes include antibiotics, anti-ulcer treatment and anti-inflammatory drugs, β-blockers, antidiabetics, antidepressants, analgesics and X-ray contrast media, among others. The developed methods can be multi-analyte [24, 33] or focused on a single type of pharmaceutical [24, 31, 38, 48], sometimes studying their TPs or metabolites [31, 38]. With regard to toxins, the cyanobacterial neurotoxins β-N-methylamino-L-alanine (BMAA) and saxitoxin are frequently studied using the HILIC approach [30, 39, 50, 51, 53]. The determination of hydrophilic saxitoxin analogues and domoic acid in environmental samples has also been reported [34, 47]. On the other hand, the drugs of abuse analysed by HILIC include cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, codeine, morphine, amphetamine, 6-acetylmorphine, among others [19, 20, 35, 48, 54].

Recent applications also include the determination of artificial sweeteners in environmental waters [22, 23]. HILIC applications have also been reported for pesticides [42], industrial related chemicals such as surfactants [55], melamine [45, 52] and acrylamide [43], and estrogens [32]. The studies of Chao et al. [28] on hydroxylated fullerenes and Rodin et al. [49] to determine methylphosphonic acid are examples of less common applications of HILIC but very interesting and promising.

The technique preferred for the sample preparation of environmental waters when using HILIC approaches is the SPE while algae, cyanobacteria or other samples are commonly extracted with acidic aqueous/organic mixtures using agitation or sonication, followed or not by a SPE clean-up step. Among the SPE sorbents available, the most frequently used is the Oasis HLB from Waters. Mixed-mode sorbents were used together with HILIC separation for compounds with ionic properties, thanks to the capability of these sorbents to retain compounds trough hydrophobic interactions as well as

Table 3. Type of analytes and matrices studied and MS instrumentation used in environmental applications of HILIC.

	Analytes	Column	Matrix	MS analyzer	Ref.
Pharmaceuticals	pharmaceuticals and illicit drugs	Ascentis Express HILIC	river and wastewaters	ESI-MS	[48]
	antibiotics	Altima HP	river water, liquid manure and run-off samples	APCI-QqQ	[27]
	pharmaceuticals	ZIC-HILIC	surface and wastewaters	ESI-Qtrap	[24]
	cytostatics	ZIC-HILIC	hospital wastewaters	ESI-QqQ, ESI-LTQ-Orbitrap MS	[56]
	pharmaceuticals	Luna HILIC	surface and wastewaters	ESI-QqQ	[60]
	antibiotics	Nucleoshell HILIC	wastewaters	ESI-QqQ	[33]
	pharmaceuticals and TPs	Acquity UPLC BEH Amide	ultrapure water	ESI-QTOF	[38]
	pharmaceuticals and TPs	Poroshell 120 EC-C_{18} and ZIC-HILIC (in series)	ultrapure water, synthetic hard drinking water and wastewater	ESI-TOF	[31]
	pharmaceuticals	ZIC-HILIC	surface waters	ESI-QqQ	[58]
	gadolinium contrast media	ZIC-cHILIC	tap water	ICP-MS	[17]
	pharmaceuticals	ZIC-HILIC	hospital wastewaters	ICP-MS	[59]
	iodinated X-ray media	ZIC-HILIC	wastewaters	ESI-QqQ	[29]

Table 3. (Cont.).

	Analytes	Column	Matrix	MS analyzer	Ref.
Toxins	β-N-methylamino-L-alanine (BMAA, neurotoxin)	ZIC-HILIC	cyanobacteria	ESI-IT-MS, ESI-QqQ	[50]
	saxitoxin (neurotoxin)	TSK-gel Amide-80	algae	ESI-IT-MS/MS	[39]
	β-N-methylamino-L-alanine (BMAA, neurotoxin)	ZIC-HILIC	river water, biofilm, cyanobacteria	ESI-QqQ	[53]
	saxitoxin analogues (neurotoxins)	TSK-gel Amide-80	algae and cyanobacteria	ESI-QqQ, ESI-QqTOF	[34]
	toxins	ZORBAX SB-C18 and Venusil HILIC (in series)	algae	ESI-TOF	[30]
	domoic acid (toxin)	ZIC-HILIC	lagoon and sea water	ESI-QqQ	[47]
	cylindrospermopsin (toxin)	Kinetex HILIC	aquatic organisms	ESI-QqQ	[44]
Drugs of abuse	cocaine and metabolites	ZORBAX Rx-SIL HILIC	surface and wastewaters	ESI-IT-MS/MS	[20, 54, 57]
	drugs of abuse	Luna HILIC	surface and wastewaters	ESI-QqQ	[19]
	cocaine and metabolites	Restek Ultra IBD phase (polar-embedded alkyl)	wastewaters	ESI-QqQ, ESI-MSD	[35]
	drugs of abuse	Restek Viva PFPP	wastewaters	ESI-QqQ	[18]

Table 3. (Cont.).

	Analytes	Column	Matrix	MS analyzer	Ref
Sweeteners	artificial sweeteners	Kinetex HILIC	wastewaters	ESI-QqQ	[23]
	artificial sweeteners	Luna HILIC	surface and wastewaters	ESI-QqQ	[40]
	artificial sweeteners	Syncronis	river and wastewaters	ESI-Orbitrap	[22]
Other	estrogens	Luna C_{18} and ZIC-HILIC (in series)	river water	ESI-Qtrap	[32]
	hydroxylated fullerenes	XBridge amide	ultrapure water	ESI-QqQ	[28]
	acrylamide	Atlantis HILIC	drinking and surface waters	ESI-QqQ	[43]
	melamine	Atlantis HILIC	fish and shrimp	ESI-QqQ	[52]
	melamine	ZIC-HILIC	crop, soil and irrigation water samples	ESI-QqQ	[45]
	surfactants	Atlantis HILIC	ultrapure water	ESI-QTOF	[55]
	pesticides	Atlantis HILIC	river water	ESI-QqQ	[42]
	herbicides and metabolites	Nucleodur HILIC	river water	ESI-QqQ	[41]
	methylphosphonic acid (chemical weapon metabolite)	Luna HILIC	well, river and tap water	ESI-Qtrap	[49]

ionic interactions [18, 19, 24]. Other approaches such as activated carbon [42], sorbents prepared in-house [48] or coupling of cartridges [56] have also been used. As previously stated, the main feature linked to HILIC is the possibility of injecting extracts obtained during the sample

treatment steps directly to the HILIC separation system.

The treatment procedure selected for any sample has a great influence on the ME observed in MS-based detectors, because the properties of the final solution to be injected in the chromatographic instrument will affect the ionization and the clean up strategies used should contribute to the elimination of interferences. The type of ionization source has also proved to have an effect on the MS response. It has been claimed that the use of HILIC helps to improve ionization in the interface between the LC instrument and the MS detector because the use of high contents of organic solvents facilitates the desolvation process [2, 3, 9]. Because other parameters apart from the LC conditions affect the ME commonly observed for complex matrices such as environmental waters, it is difficult to assure whether the use of HILIC has contributed or not to its reduction. Similarly, the comparison among the HILIC methods developed can be a challenge as different sample treatment procedures and MS conditions are used. Nevertheless, some discussion on how ME is assessed and the results observed for the studies reviewed can be discussed. Most of the MS detectors coupled to HILIC separations are based on tandem MS and they are also equipped with ESI sources, because this ion source is more adequate for polar compounds. In some cases, APCI was tested achieving lower ME but also lower sensitivity [43]. Table 3 shows how instruments based on ESI-QqQ are the detectors mostly coupled with HILIC. However, instruments using ion trap (IT or Q-trap) are also reported. In some cases, HILIC has been coupled to HRMS to take advantage of its powerful identification [22, 34, 38, 56], including instruments based on Orbitrap, linear ion trap (LTQ)-Orbitrap, time of flight (TOF) and Q-TOF.

Among HILIC methods applied to environmental samples, some reported low ME values while other reported high ME affecting the MS response. In several cases, some discussion was made but data was not shown or ME was simply not subjected to discussion. For instance, Rodin et al. [49] reported no ME (-2% in average) for methylphosphonic acid in surface water samples while van Nuijs et al. [19] observed ME values up to -51% for drugs of abuse in a similar matrix. Echeverría et al. [29] observed high ME values for X-ray contrast

media in wastewater samples even after testing several strategies to reduce it. They claimed that analytes showed higher response when working in ultrapure water but signal decreased when analyzing environmental samples. Different studies determining BMAA in cyanobacteria have observed different values of ME, being supposedly low [51] according to the recoveries reported, moderate (-40 %) [53] or exhibiting suppression of 5 times the response [50]. When discussing the results, there is little attention to the advantages that HILIC really offers regarding ME, because the comparison of this LC mode with others such as RPLC is frequently done during optimization with ultrapure water in terms of retention and separation. In order to properly evaluate HILIC performance on this matter, comparison with another LC separation must be done using the same complex matrix. Peru et al. [27] observed very high ME for spectinomycin (up to 100 % of enhancement) when using a RPLC approach while in the HILIC approach it was negligible, because the lack of retention in RPLC caused co-elution with polar interferences in the matrix. Ordóñez et al. [21] compared a RPLC method with a HILIC approach in terms of the ME observed for an influent wastewater sample, finding better results for the RPLC separation (-23 to 3 %) versus HILIC (-93 to -31 %) observing high signal suppression for saccharine and sucralose (-93 and -90%, respectively). In summary, HILIC has proved to show excellent results within the environmental field for retaining and separating polar and hydrophilic compounds, however, its advantages regarding ME are not frequently thoroughly discussed.

5 Conclusions

Within the environmental field, HILIC has proved to be an excellent alternative for the determination of several contaminants whose polar properties caused problems in retention, separation and detection when using conventional RPLC methods. Bare silica, zwitterionic, amide and diol are the stationary phases mostly used for environmental applications using ACN/aqueous mobile phases usually containing ammonium formate and acetate buffer solutions. HILIC is mostly coupled with QqQ analyzers using ESI sources (adequate for the polar character of a great number of contaminants), thanks to their suitable selectivity and sensitivity,

achieving low quantification limits for these complex samples.

Selected examples showed less matrix effect and enhanced sensitivity when using HILIC; however, discussion addressing schematic comparison in this regard between both RPLC and HILIC is often missing.

Recent progress is focused on benefiting from the complementary selectivity of RPLC and HILIC either by coupling columns in series or injecting the samples in two developed methods.

HILIC has established itself as a promising technique for the determination of polar compounds in environmental samples, which could potentially improve the chromatographic separation and sensitivity to better quantify the contaminants in these highly complex matrices.

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UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

1.2. Mixed-mode ion-exchange polymeric sorbents for solid-phase extraction

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

Recent progress in the development of new sorbents for solid-phase extraction has focused on increasing their capacity and selectivity. As mentioned previously, the development of hypercrosslinked polymeric sorbents with ultra-high surface areas (> 1,000 m^2/g) has improved the capacity of SPE sorbents by providing more binding sites. The introduction of polar functionalities to polymeric sorbents has also contributed in this respect, improving the capacity of the sorbents for polar compounds. Sorbents focused on improving selectivity towards a specific group of compounds have also been developed, with the best example being molecularly imprinted materials (MIPs). MIPs are prepared using a template of the target analyte which creates specific cavities within the polymeric network that is highly specific for the target analyte or very structurally similar compounds.

The introduction of mixed-mode ion-exchange polymeric sorbents responded to the lack of sorbents improving both capacity and selectivity towards ionizable compounds. They are prepared by introducing ionic functional groups to polymeric supports, which may or may not have polar functional groups, producing sorbents capable of retaining hydrophobic compounds through reversed-phase interactions, ionizable compounds through ion-exchange interactions and polar compounds through several mechanisms, such as hydrogen bonding or Van der Waals forces [109].

These sorbents are classified according to the ionic functional groups introduced to the polymeric backbone, being cation or anion exchangers with strong or weak properties. Strong cation-exchangers (SCX) usually have sulfonic acid groups while weak cation-exchangers (WCX) have carboxylic acid groups. Anion-exchangers are normally obtained by modifying polymers with different types of amines, having strong anion-exchange properties (SAX) if the amine groups are quaternary, or weak anion-exchange properties (WAX) if the groups attached are tertiary, secondary or primary amines [56]. Strong ion-exchangers provide permanently charged moieties regardless of the pH and solvent conditions used. Thus, the ionic interactions between the sorbent and the compounds can be established or disrupted by controlling the state form of the target analytes, selecting the adequate pH conditions

according to their pK_a values. Permanently charged analytes can be strongly retained on strong ion-exchangers requiring concentrated eluting solutions or be irreversible retained. In this respect, ion-exchangers with weak properties are more suitable for these types of compounds, as they are charged on a specific pH range, having a pK_a value associated. Therefore, by controlling the pH conditions, the ionic interactions can be switched on or off by protonating and deprotonating the functional groups of the sorbents [63].

These sorbents can be either "universal" or very selective depending on whether or not a washing step is used. They can be universal because the list of compounds that can be extracted from the samples is larger compared to other sorbents, thanks to the enhanced retention toward ionic compounds given by the presence of ionic functionalities. Nevertheless, their selectivity can be increased towards ionic analytes if the compounds retained through interactions different from ion-exchange are rinsed from the sorbents, using washing steps with organic solvents to disrupt these interactions. Thus, ionic compounds are eluted in the elution fraction separately from neutral ones [109].

1.2.1. Development of mixed-mode ion-exchange sorbents

Nowadays, there are several commercial mixed-mode ion-exchange polymeric sorbents available, and also different research groups have developed them in-house and used them in several applications. Table 2 summarizes the most widely used mixed-mode ion-exchange polymeric sorbents, classified by type of ionic-exchanger. Probably, the group of sorbents most widely used is the Oasis brand, as they have been developed from the well-known Oasis HLB as starting material, based on poly(vinylpyrrolidone-divinylbenzene) (PVP-DVB). Chloromethylation of Oasis HLB provides polymeric particles with primary chloride groups on the surface susceptible to react and produce functionalized sorbents with different groups. From these reactions, four mixed-mode ion-exchangers were synthetized and commercialized with properties of strong cation-exchange (Oasis MCX), strong anion-exchange (Oasis MAX), weak cation-exchange (Oasis WCX) and weak anion-exchange (Oasis WAX). Oasis MCX

was actually synthesized directly from Oasis HLB through sulfonation of the aromatic rings in the polymer particles. For this purpose, concentrated H_2SO_4 was slowly added to the starting material and the ratio grams H_2SO_4/grams Oasis HLB and reaction time were optimized. The rest of the Oasis materials were synthesized from the chloromethylated material by means of substitution reactions with the desired amines or oxidation of the chloride group. To synthesize the Oasis MAX material, different amines were tested including dimethylethylamine (DMEA), diethylmethylamine (DEMA), dimethylbutylamine (DMBA) and trimethylamine (TEA), with higher ion-exchange capacity being displayed by the polymer modified with DMBA. In the case of Oasis WAX, the amines tested were dibutylamine, diisopropylamine, morpholine and piperazine, with the last of these selected as it yielded higher ion-exchange capacity. Finally, for the Oasis WCX, the chloromethylated Oasis HLB was reacted with a solution of 30% H_2O_2 to form the carboxylic acid moieties [63]. According to the manufacturer, Oasis MCX is selective for bases and Oasis MAX is selective for acids, while Oasis WCX and Oasis WAX are capable of retaining bases and acids with strong properties, respectively.

Table 2. List of several mixed-mode ion-exchange polymeric SPE sorbents commercially available or in-house synthesized.

Type	Name	Functional groups	Polymeric support
Strong cation-exchanger (SCX)	Oasis MCX (Waters)	sulfonic acid	PVP-DVB
	Strata X-C (Phenomenex)	sulfonic acid	PS-DVB modified with vinylpyrrolidone
	Bond Elut Plexa PCX (Agilent)	proprietary	PS-DVB modified with hydrophilic groups
	Supel™-Select SCX (Supelco)	sulfonic acid	PS modified with hydrophilic groups
	Evolute Express CX (Biotage)	sulfonic acid	PS-DVB modified with non-ionizable hydroxyl groups
	ExtraBond ECX (Scharlau)	proprietary	PS-DVB

Table 2. (Cont.).

Type	Name	Functional groups	Polymeric support
Strong cation-exchanger (SCX)	Cleanert PCX (Agela Technologies)	proprietary	PS-DVB
	Speedisk H2O-Philic SC-DVB (J.T. Baker)	sulfonic acid	
	HXLPP-SCX (in-house)	lauroyl sulfate	hypercrosslinked VBC-DVB
	AMPSA-HEMA-PETRA (in-house)	sulfonic acid	AMPSA-HEMA-PETRA
	Sufonated HEMA-DVB (In-house)	sulfonic acid	HEMA-DVB
Strong anion-exchanger (SAX)	Oasis MAX (Waters)	quaternary amine (DMBA)	PVP-DVB
	Strata X-A (Phenomenex)	quaternary amine (DMBA)	PS-DVB modified with vinylpyrrolidone
	Bond Elut Plexa PCX (Agilent)	proprietary	PS-DVB modified with hydrophilic groups
	Supel™-Select SAX (Supelco)	quaternary amine	PS modified with hydrophilic groups
	Evolute Express AX (Biotage)	quaternary amine	PS-DVB modified with non-ionizable hydroxyl groups
	ExtraBond EAX (Scharlau)	proprietary	PS-DVB
	Cleanert PAX (Agela Technologies)	proprietary	PS-DVB
	Speedisk H2O-Philic SA-DVB (J.T. Baker)	quaternary amine	
	NVIm-DVB (in-house)	imidazole	NVIm-DVB
	HXLPP-SAX	quaternary amine (DMBA)	hypercrosslinked VBC-DVB
Weak cation-exchanger (WCX)	Oasis WCX (Waters)	carboxylic acid	PVP-DVB
	Strata X-CW (Phenomenex)	carboxylic acid	PS-DVB modified with vinylpyrrolidone

Table 2. (Cont.).

Type	Name	Functional groups	Polymeric support
Weak cation-exchanger (WCX)	Evolute Express WCX (Biotage)	carboxylic acid	PS-DVB modified with non-ionizable hydroxyl groups
	Cleanert PWCX (Agela Technologies)	proprietary	amino PS-DVB
	HXLPP-WCX	carboxylic acid	VBC-DVB-MAA
Weak anion-exchanger (WAX)	Oasis WAX (Waters)	piperazine	PVP-DVB
	Strata X-AW (Phenomenex)	diamine	PS-DVB modified with vinylpyrrolidone
	Evolute Express WAX (Biotage)	primary/secondary amine	PS-DVB modified with non-ionizable hydroxyl groups
	Cleanert PWAX (Agela Technologies)	proprietary	PS-DVB
	HXLPP-WAX (in-house)	1,2-ethylenediamine or piperazine	hypercrosslinked VBC-DVB

DVB: divinylbenzene; PVP: vinylpyrrolidone; PS: polystyrene; NVIm: N-vinylimidazole; VBC: vinylbenzylchloride; MAA: methacrylic acid; AMPSA: 2-acrylamido-2-methylpropanesulphonic acid; HEMA: 2-hydroxyethyl methacrylate; PETRA: pentaerythritol triacrylate.

Other brands also used existing polymeric sorbents with reversed-phase and/or hydrophilic properties as the support for functionalized mixed-mode ion-exchangers. For instance, the Strata-X sorbents with ion-exchange properties from Phenomenex are based on the Strata-X backbone of the polystyrene-divinylbenzene copolymer (PS-DVB) modified with vinylpyrrolidone groups. From this material, Strata-X-C (with SCX properties), Strata-X-A (SAX), Strata-X-CW (WCX) and Strata-X-AW (WAX) were developed. The functional groups introduced to the polymer particles are very similar to the Oasis sorbents, with the exception of Strata-X-AW, which is modified with ethylenediamine (EDA) groups.

Bond Elut Plexa based on a PS-DVB backbone is also available with ion-exchange properties as Plexa PCX (SCX) and Plexa PAX (SAX). Sigma-

Aldrich has also polystyrene (PS) networks modified with hydrophilic properties and functionalized with SCX and SAX moieties, commercialized as Supel-Select SCX and Supel-Select SAX, respectively. Biotage developed the brand Evolute which has available several mixed-mode ion-exchangers with SCX, SAX, WCX and WAX properties. The support for these materials is PS-DVB incorporating nonionizable hydroxyl groups. Other examples of brands developing these sorbents include ExtraBond (Scharlau), Cleanert (Agela Technologies) and Speedisk (J.T. Baker).

Based on the studies reporting the synthesis and application of in-house mixed-mode sorbents, our research group developed a large number of sorbents with good results. Similar to commercial sorbents, some of them were based on macroreticular polymers, while others used hypercrosslinked (HXL) particles as the support. The process to synthesize HXL materials was introduced by Davankov [110], consisting of a Friedel-Crafts reaction with external electrophiles, which promotes the formation of bridges between neighbouring aromatic rings, increasing the surface area and the micropore content of the starting polymer. These materials are also prone to solvent uptake of both polar and nonpolar solvents, giving them excellent wettability [111]. Sherrington's group developed a method to produce HXL polymers via internal electrophiles using vinylbenzylchloride (VBC) as a comonomer to synthesize the starting polymer, which had pendent chloromethyl groups that acted as electrophiles capable of reacting with the aromatic benzene rings [112]. The HXL material obtained with ultra-high surface area has residual chloromethyl groups susceptible to substitution reactions, which are very useful for producing sorbents with mixed-mode ion-exchange properties.

Examples of in-house sorbents developed in our group and based on macroreticular polymers include a copolymer of N-vinylimidazole-divinylbenzene (NVIm-DVB) synthesized by suspension polymerization [113], a copolymer of 2-acrylamido-2-methylpropanesulphonic acid (AMPSA), 2-hydroxyethyl methacrylate (HEMA) and pentaerythritol triacrylate (PETRA) and a HEMA-DVB copolymer modified with H_2SO_4 [114]. The NVIm-DVB sorbent was evaluated as an anion-exchanger and compared with Oasis MAX and Oasis WAX, with the sorbent being found

to behave as a SAX material rather than a WAX material, giving results similar to those obtained with Oasis MAX [113]. Both the AMPSA/HEMA/PETRA and sulfonated HEMA-DVB sorbents, had sulfonic acid groups capable of promoting SCX interactions.

Using these HXL polymers as the support, sorbents with SCX [115, 116], SAX [117], WCX [118] and WAX [119] properties have been developed by our group. For the synthesis of HXLPP-SCX materials supported on hypercrosslinked VBC-DVB via precipitation polymerization (PP), the introduction of sulfonic acid groups was attempted using acetyl sulfate and lauroyl sulfate as reagents. From the results, it was found that lauroyl sulfate yielded sorbents with higher sulfonic group content [115]. In another study, the introduction of sulfonic groups using lauroyl sulfate was also tested on particles obtained by non-aqueous dispersion polymerization (NAD) and compared with particles produced by PP [116]. HXLPP-SAX materials were synthesized introducing the amino groups (using DMBA) previous to the HXL reaction. The amination to the HXL polymers was less efficient because the pendent chloromethyl groups were hindered by the bulky DMBA. Results obtained for these materials were similar to or better than Oasis MAX and SampliQ SAX [117]. An HXLPP-WCX sorbent was developed from the copolymerization of VBC, DVB and methacrylic acid (MAA) to yield a polymer with carboxylic acid groups, which was subsequently subjected to the HXL reaction [118]. This material proved to be more selective and offer higher capacity than Oasis WCX and Strata-X-CW. HXLPP-WAX materials were based on hypercrosslinked VBC-DVB modified with 1,2-ethylenediamine (EDA) and piperazine groups [119]. In this study, the HXLPP-WAX sorbents were superior to the commercial sorbents Oasis WAX and Strata-X-AW. This material was later applied to develop an on-line SPE method [120]. In general, these sorbents proved to yield great recoveries for the model compounds selected for their structure and showed promising results which allowed the development of methods for the analysis of environmental samples.

In recent years, other research groups have reported the synthesis and application of in-house sorbents with ion-exchange mixed-mode properties.

For instance, Wang *et al.* [121] developed a polymeric cation-exchanger with EDA moieties which exhibit Cu^{2+} adsorption. The synthesis consisted of several steps, including the acylation of PS-DVB microspheres followed by the atom transfer radical polymerization of glycidylmethacrylate to obtain PS-DVB-g-PGMA particles with epoxy groups. Then, the epoxy groups were ring-opened by ethylenediamines, obtaining a polymer with amino groups (PS-DVB-g-PGMA-EDA).

Other recently developed sorbents are silica-based, some of which have functional groups with interesting properties. For example, Zhu *et al.* [122] and Zhang *et al.* [123] have developed sorbents with cation-exchange properties (containing carboxyl and n-dodecyl groups using click chemistry) and anion-exchange properties (containing calixarene groups), the latter capable of hosting supramolecules. Other types of supports like multi-walled carbon nanotubes [121] or magnetic particles [40] have also been functionalized with ion-exchange properties giving rise to new mixed-mode sorbents.

1.2.2. Influence of the SPE protocol

The selection of the most suitable conditions used in each step of the SPE procedure is very important because they influence the performance of the sorbent selected. Naturally, the formation of ionic interactions between the functional groups of the sorbent and the analytes strongly depends on the pH value selected for the loading step, as both the sorbent and the analytes must be in their ionic form. As previously discussed, in strong ion-exchangers, the ionic functional groups are always charged, so the ionization state of the analytes must be switched to promote formation or disruption of ionic interactions with the sorbent. In the case of weak ion-exchangers, the charge of both the sorbent and the analytes can be controlled by changing the pH taking into account the pK_a values of the functional groups attached to the polymeric backbone and the solutes [109]. Table 3 describes the typical SPE protocol required to promote selective retention of acidic or basic compounds commonly used for each type of ion-exchanger, as well as the

washing conditions that may help to eliminate interferences and the conditions to elute the analytes by disrupting the ionic interactions.

Table 3. Typical SPE protocols for the SCX, SAX, WCX and WAX type mixed-mode sorbents.

SPE steps	SCX	SAX	WCX	WAX
Conditioning	Organic solvent and aqueous conditions similar to sample			
Loading	Acidic sample (pH < pK_a of basic compound)	Neutral or basic sample (pH > pK_a of acidic compound)	Neutral sample (pK_a acidic group sorbent < pH < pK_a basic compound)	Neutral sample (pK_a basic group sorbent > pH > pK_a acidic compound)
Washing	Acidic aqueous solution and/or pure organic solvent	Basic aqueous solution and/or pure organic solvent	Basic aqueous solution and/or pure organic solvent	Acidic aqueous solution and/or pure organic solvent
Elution	Basic solution in pure organic solvent	Acidic solution in pure organic solvent	Acidic solution in pure organic solvent	Basic solution in pure organic solvent

Similar to conventional polymeric sorbents, the conditioning is performed with pure organic solvent followed by an aqueous solution with similar conditions to the sample, with the aim of preparing the sorbent for the reception of the sample. In order to select a suitable pH for loading the sample, the pK_a values of the analytes should be taken into account, together with those corresponding to the groups on the sorbents with weak properties. For loading samples in the SCX sorbent, only the pK_a values of the basic analytes must be considered. As basic organic compounds can be deprotonated at high pH values (pK_a values ~7 to 10), an acidic pH is always suitable for loading the sample. Similarly, when using SAX sorbents, acidic organic compounds (pK_a values ~2 to 5) must be negatively charged to interact with the permanently charged amine groups of the sorbent, and so neutral or basic loading pH values are adequate. For exchangers with weak properties, the selection of a neutral value (pH ~7) is often suitable because

it prevents the deprotonation of the amine groups (both in the sorbent and the analytes) and the protonation of the acidic groups.

The washing step must be selected according to the requirements of the extraction. To fully exploit the selectivity of these sorbents, a washing step with a pure organic solvent can be included to eliminate neutral interferences or compounds with the same charge as the sorbents. Usually, the preferred solvent for this purpose is MeOH, because it is suitable for solubilizing polar compounds and has proper elution strength. Aqueous solutions can also be included during the washing step as indicated in Table 3, for the purpose of eliminating salts and water-soluble compounds poorly retained by the sorbent through reversed-phase interactions [109]. Aqueous solutions of 5% HCOOH or 5% NH_4OH are normally recommended for this type of clean-up. However, this washing step should be applied with care as it can sometimes affect retention. For instance, when using a WCX sorbent, basic analytes may shift to their neutral state when washing with a basic solution, leading to a decrease in their retention. An example of a study using an aqueous washing step is the method for the extraction of illicit drugs from wastewaters [124], where the SCX cartridge is washed with ultrapure water adjusted at pH 4.5. Other detailed examples will be listed in further sections.

During the elution step, the ionic interactions are disrupted by passing an organic solution at a suitable pH. An organic solution with suitable strength must be used to elute the ionic compounds that had been turned to neutral due to the acidic/basic additive. Manufactures often recommend the use of 5% HCOOH or 5% NH_4OH in MeOH for this purpose. For strong exchangers, the analytes must be turned into their neutral form and, as such, basic elutions are performed in the SCX sorbents, while acidic solutions are used for SAX sorbents. In the case of weak exchangers, acidic or basic solutions can be adequate because either the groups of the sorbent or the analytes can be switched into their neutral form. For instance, the carboxylic acid groups in a WCX sorbent can be protonated by using an acidic solution, but retained basic compounds can also be deprotonated with a basic solution. The selection between these options can be made according to the pK_a values or experimentally comparing the obtained recoveries. As an

example of a typical SPE protocol, a procedure to extract a neurotoxin from environmental samples using a SCX sorbent included loading at pH 3, washing with a 0.1 M HCl followed by pure MeOH and finally eluting with a 5% NH_4OH in MeOH solution [125].

Alternatively, these ion-exchangers can be used to retain ionic interferences and rinse neutral target analytes during the washing step with organic solvents. For instance, Carpinteiro *et al.* [126] used Oasis MAX cartridges to retain ionic interferences from river water and wastewaters while rinsing the neutral analytes corresponding to four benzotriazolic compounds and six benzothiazole derivatives. For this purpose, the cartridges were conditioned with 5 mL MeOH followed by 5 mL ultrapure water, after which the samples were loaded (pH not indicated). Subsequently, the analytes were eluted with 5 mL of a MeOH/acetone (7:3, v/v) mixture. According to the authors, this procedure obtained clearer extracts because acidic interferences were retained in the cartridge separately from target analytes. It can be seen that, by fully understanding the capabilities of these sorbents and the nature of the target analytes, the SPE protocol can be designed for different purposes.

1.2.3. Application of mixed-mode ion-exchange sorbents

A great number of studies can be found in the literature focused on applications of mixed-mode ion-exchange polymeric sorbents. Most of them exploit the selectivity of these sorbents to eliminate neutral interferences of the matrices or, in contrast, take advantage of the enhanced capability of these materials to retain acidic or basic compounds. Table 4 includes a selection of the most commonly cited studies, as well as the most recent ones using these sorbents, indicating the target analytes and the matrices of interest.

Table 4. Selection of the most commonly cited and the most recent applications of ion-exchange mixed-mode sorbents organized by type of matrix.

Type of Matrix	Analytes	Matrix	Cartridge	Ref.
Environmental	Beta-lactam antibiotics	Wastewaters	Oasis MAX	[127]
	Pharmaceuticals and drugs of abuse	Surface water	Oasis MCX	[128]
	Melamine	Fish and shrimp	Oasis MCX	[129]
	Pharmaceuticals and metabolites	Wastewaters and surface waters	Oasis MCX	[130]
	Pharmaceuticals, personal care products and drugs of abuse	Wastewaters and river water	Oasis MCX	[131]
	Pharmaceuticals	Wastewaters and river water	HXLPP-WAX (in-house)	[119]
	Tetracyclines and degradation products	Wastewaters and river water	Oasis MAX	[132]
	Pharmaceuticals	Wastewaters and river water	HXLPP-WCX (in-house)	[118]
	Endocrine-disrupting compounds	Surface water	Oasis MCX	[133]
	Drugs of abuse	Wastewaters	Oasis MCX	[124]
	Pharmaceuticals	River water and wastewaters	HXLPP-SAX (in-house)	[117]
	Neurotoxin	River waters, biofilm and cyanobacteria	Oasis MCX	[125]

Table 4. (Cont.).

Type of Matrix	Analytes	Matrix	Cartridge	Ref.
Environmental	Drugs of abuse	River water and wastewaters	Oasis WCX	[134]
	Antimycotic drugs	River water and wastewaters	Oasis MCX	[135]
	Perfluorinated compounds	Fish, vegetables and soil	Oasis WAX	[136]
	Herbicides	Stormwater samples	Oasis MAX	[137]
	Pharmaceuticals and drugs of abuse	Wastewaters	HXLNAD-SCX, HXLPP-SCX (in-house)	[116]
	Pharmaceuticals and drugs of abuse	Wastewaters	AMPSA/ HEMA/ PETRA, Sulfonated HEMA/DVB (in-house)	[114]
	Pharmaceuticals	River water and wastewaters	Oasis MAX	[138]
	Perfluorinated compounds	Mussel and fish tissue and liver samples	Evolute-WAX coupled in tandem with Envi-Carb	[139]
Biological	Beta-agonists	Urine and liver	Bond-Elut Certify	[140]
	Drugs of abuse	Human plasma	Oasis MCX	[141]

Table 4. (Cont.).

Type of Matrix	Analytes	Matrix	Cartridge	Ref.
Biological	Pharmaceuticals	Rat plasma	Oasis MCX	[142]
	Melamine	Muscle tissue	Strata-X-C	[143]
	Cardiovascular drugs	Post-mortem whole blood	Oasis MCX	[144]
	Antidepressants and metabolites	Human oral fluid and plasma	Oasis MCX	[145]
	Drugs of abuse	Human urine	Oasis MCX	[146]
	Psychotropic drugs and metabolites	Human plasma	Oasis MCX	[147]
	Drugs of abuse	Human urine	Clean Screen DAU	[148]
	Ethyl glucuronide	Hair	Oasis MAX	[149]
	Melamine	Rat plasma and organs	Oasis MCX	[150]
	Proteins	Human plasma	Bond Elut Plexa	[151]
	Drugs of abuse and metabolites	Whole blood	Isolute Confirm HCX	[152]
	Drugs of abuse	Serum	Chroma-bond Drug	[153]
	Flame retardants and metabolites	Human urine	Strata-X-AW	[154]
	Nicotine and metabolites	Human plasma	Oasis MCX	[155]
	Drugs of abuse	Human blood	Evolute CX	[156]
	Amyloid beta peptides	Human cerebrospinal fluid	Oasis MCX	[157]
	Doping drugs	Human urine	Strata-X-CW	[158]

Table 4. (Cont.).

Type of Matrix	Analytes	Matrix	Cartridge	Ref.
Biological	Selective serotonin reuptake inhibitors and metabolites	Human plasma	Oasis MCX	[159]
	Doping hormones metabolites	Rat urine	Strata-X-CW	[160]
	Hypnotics and metabolites	Human urine and blood	Bond Elut Plexa PCX	[161]
	Bisphenol AF	Rat tissues, serum, urine and faeces	Oasis MAX	[162]
	Antibiotics	Human plasma	Oasis MCX	[163]
	Desmosine and isodesmosine	Human plasma and urine	Oasis MCX	[164]
	Doping drugs	Human urine	Bond Elut Plexa PCX	[165]
	Drugs of abuse	Human serum	Resprep Drug Prep I	[166]
	Urine metabolites	Human urine	Strata X-AW, Strata X-C	[167]
	Propofol glucuronide (metabolite of the anaesthesia drug propofol)	Hair	Oasis MAX	[168]
	New psychoactive substances	Vitreous humour, pericardial fluid, whole blood	Oasis MCX	[169]

Table 4. (Cont.).

Type of Matrix	Analytes	Matrix	Cartridge	Ref.
	Metabolites of organophosphate flame retardants	Human urine	Strata-X-AW	[170]
	Non-steroidal anti-inflammatory drugs (NSAID)	Human urine, surface, river and ground waters	Oasis MAX, in-house imidazole-based polymers	[171]
Food	Beta-agonists	Bovine and porcine urine, feed and hair	Bond-Elut Certify	[172]
	Fungicides	Wine	Oasis MAX	[173]
	Anthocyanins	Fruits and vegetables	Oasis MCX	[174]
	Antimicrobial drugs and metabolites	Bovine, equine and porcine liver	Oasis MCX	[175]
	Isoflavones and resveratrols	Vegetable oils	Oasis WCX	[176]
	Veterinary drugs and metabolites	Bovine milk	Oasis MCX	[177]
Other	Plant hormones	Plant material	Oasis MAX	[178]
	Phytohormones	Plant tissues	HR-XC	[179]
	Surfactants	Foams and concentrates	Oasis WCX and Oasis WAX	[180]

As can be seen, most of the applications of mixed-mode ion-exchangers polymeric focus on environmental and biological matrices. Within environmental applications, surface waters and wastewaters are the matrices most treated using these types of sorbents, but applications in other types of

samples (fish, soil and biofilm samples) can also be found. The analytes extracted from these matrices are mainly pharmaceuticals and drugs of abuse with ionic properties, finding to a lesser extent but equally successful methods for other contaminants such as endocrine disrupting compounds, toxins and herbicides.

With regard to biological samples, the applications of mixed-mode sorbents have been reported in human urine, plasma, blood, oral fluid, hair and cerebrospinal and pericardial fluids, as well as rat plasma, serum, organs and tissues. Similar to environmental studies, biological applications frequently focus on the determination of pharmaceuticals and drugs of abuse. However, in these cases, the aim of the study is to monitor the levels of a compound with therapeutic relevance, identifying illicit drugs of new design, such as psychoactive drugs and doping agents, or studying metabolomic or proteomic profiles. Frequently, for these types of samples, the SPE step is introduced after a previous solid-liquid extraction of the biological matrices, in contrast to environmental waters, for instance, which can be loaded on the SPE cartridges after a simple filtration step. The potential of mixed-mode ion-exchangers has also been proven in the extraction from food matrices and plant samples. In several cases, the extraction of metabolites was also described together with the target analytes. It should also be noted that the Oasis exchangers are the most widely used sorbents, particularly those with strong properties. In general, the application of these sorbents has spread across several fields and their selective extraction achieved through effective clean-up steps has contributed to reducing the ME.

Recent studies in the environmental field have reported the elimination of interferences and therefore the reduction of the ME thanks to the use of mixed-mode sorbents. For instance, Triñanes *et al.* [138] stated that the use of Oasis MAX significantly decreased the ME obtained for the target pharmaceuticals, in comparison to the results obtained for Oasis WAX and reversed-phase sorbents. When analysing raw wastewater samples with the Oasis MAX cartridges, the ME values were between -32% and -10%, whereas, in the case of the Oasis HLB cartridges, the results obtained were between -68% and -51%. In the study by Zabaleta *et al.* [139], mixed-mode

SPE was used to extract perfluorinated compounds and eliminate interferences present in the extracts obtained after the focused ultrasound solid-liquid extraction of mussels, mullet liver and tuna tissue samples. For this purpose, a WAX exchanger (Evolute-WAX), a graphitized carbon sorbent (Envi-Carb) and the tandem combination of both were compared. Colourless and clearer extracts were obtained when combining both sorbents in-line, correcting the ME observed for some analytes. In the method reported by Casado *et al.* [135] for the determination of antimycotic drugs in environmental waters, higher selectivity and reduction of the ME was observed when comparing Oasis MCX cartridges with Oasis HLB ones. Núñez *et al.* [181] observed a reduction in the ME when cleaning fish samples using Oasis MCX cartridges in comparison to a method in which this SPE procedure was not included.

In recent studies on biological samples, discussion has also been included about the ME and the advantages of using mixed-mode sorbents. In the study by Kwak *et al.* [168] describing the determination of propofol glucuronide in hair, the ME reported was quite low (around -10%), which was attributed to the efficiency of the sample preparation procedure. During the SPE protocol, the negatively charged analyte was retained by the amine groups of the sorbent selected (Oasis MAX), allowing the introduction of a washing step with 0.01 M sodium hydroxide solution followed by pure ethyl acetate. According to the authors, the results obtained from LC-MS/MS analysis evidenced the elimination of interfering substances from the hair samples showing high selectivity for the target compound. Miliotis *et al.* [164] also proved that the SPE procedure using Oasis MCX helped to clean the sample from interferences causing ion suppression. In this study, the use of ultra-filtration membranes (30 kDa cut-off) eliminated large proteins like hemoglobin, albumin and immunoglobulins from plasma samples, but did not provide sufficient sample clean-up, with ME being observed in these filtrates. To overcome the ME and improve sensitivity, they included an SPE procedure using a C_{18} sorbent and an Oasis MCX. The authors described the overall methodology as three consecutive and orthogonal sample preparation steps based on size, hydrophobicity and charge. Interestingly, the target

analytes (desmosines) were not retained on the C_{18} sorbent when the sample was loaded on this cartridge, so the liquid collected during this loading step containing the analytes was directly percolated through the Oasis MCX. On the Oasis MCX, the desmosines were retained through ionic interactions, allowing the inclusion of an additional washing step using 50 mM HCl and MeOH. Furthermore, they proved that the omission of any of these sample preparation steps led to a loss of signal intensity.

Colin *et al.* [163] clearly emphasized the superior recoveries (92%) obtained for beta-lactams from plasma when using mixed-mode ion-exchangers, in comparison to other methods (70%) were Oasis HLB was selected. This was attributed to the additional ion-exchange capabilities of these types of materials. The introduction of the mixed-mode SPE protocol after protein precipitation procedures proved to reduce the ME, even when the washing step applied on the Oasis MCX sorbent did not contain pure organic solvent, consisting only of a 2% HCOOH aqueous solution. Because evidence suggested that the interferences were eliminated using the aqueous washing step, they probably had very polar and hydrophilic interferences that were not retained in the polymeric backbone (probably acidic compounds with low pK_a values). This idea about the nature of the interfering compounds agrees with the fact that the results in terms of the ME were similar to a study that used Oasis HLB cartridges [182], as discussed by the authors. This example demonstrates how the mixed-mode sorbents were superior thanks to their enhanced retention of ionic compounds rather than their capability in terms of eliminating interferences.

Thus, several studies have modified the recommended protocol, as can be seen in Table 5, where the SPE protocols of a selection of recent studies are shown, describing the conditions of the loading, washing and elution steps. In every case, it depends on the objective proposed for the SPE (enhanced retention of ionic compounds or selectivity), the nature of the analytes and the interferences present in the matrix. Comparison of ion-exchangers with reversed-phase sorbents or between SPE protocols with and without washing steps are experiments that can be very helpful to gain insights into the best conditions to benefit from these sorbents.

Table 5. SPE protocols of selected recent studies in the environmental, biological and food fields.

Type sorbent	Analyte/ Matrix	Load Vol/pH	Wash Vol/solvent	Elution Vol/solvent	Ref.
SCX	Toxin/River, biota	pH 3 (HCOOH)	1) 1 mL 0.1 M HCl, 2) 2 mL MeOH	3 mL 5% of NH_4OH in MeOH	[125]
	Drugs of abuse/ WW	200 mL or 500 mL WW pH 4.5	10 mL H_2O pH 4.5	1) 2 mL MeOH, 2) 4 mL 5% NH_4OH in MeOH	[183]
	Serotonin reuptake inhibitors/ Plasma	600 µL supernatant (ACN precipitation)	1) 1,000 µL 1M citric acid, 2) 500 µL MeOH	2 x 100 µL 90:10 MeOH:25% NH_4OH	[159]
	Psycho actives/ Human fluids	Sample in 0.1 M phosphate buffer pH 4.4	1) 2 mL H_2O, 2) 2 mL 0.1 M HCl, 3) 70:30 CH_2Cl_2:MeOH, 4) 3 mL hexane	3 mL 78:20:2 CH_2Cl_2: 2-propanol: NH_4OH	[169]
	Anthocyanins/ Fruits and vegetables	SLE extract in 0.1% TCA in H_2O	1) 12 mL 0.1% TFA in H_2O	1) 12 mL 0.1% TFA in MeOH, 2)6 mL 1% NH_4OH in MeOH and in 40:60 H_2O:MeOH	[174]
	Veterinary drugs/Bovine milk	LLE extract in 0.1 M HCl	1) 2 mL 0.1 M HCl, 2) 2 mL H_2O, 3) 2 mL 15% MeOH in H_2O	3 mL 10% NH_4OH in 2:1 ACN: methyl tert-butyl ether	[177]

Table 5. (Cont.).

Type sorbent	Analyte/ Matrix	Load Vol/pH	Wash Vol/solvent	Elution Vol/solvent	Ref.
SAX	NSAID/ Urine	1 mL urine	1) 1 mL 50 mM NaCOOH pH 5.5, 2) 1 mL MeOH	1 mL 10% HCOOH in MeOH	[171]
	Herbicides/ Storm-water samples	250 mL sample pH 7 (HCl)	10 mL 50 mM CH_3COONa	1) 12 mL MeOH (neutral and basic herbicides), 2) 12 mL 5% HCOOH in MeOH	[137]
	Propofol glucuronide/Hair	~1 mL basic digested sample	1) 2 mL 0.01M NaOH, 2) 2 mL ethyl acetate	2 mL 2% HCOOH in ethyl acetate	[168]
	Fungicides/ Wine	20 mL diluted sample	5 mL 5% NH_4OH in H_2O (w/v)	1 mL MeOH	[173]
WCX	Drugs of abuse/ River and WW	10 mL pH 7	0.5 mL MeOH	0.5% HCOOH and 10% ACN in H_2O (pH 2.5)	[134]
	Doping hormones/ Urine	2 mL urine	1) 1 mL H_2O, 2) 1 mL MeOH	5% HCOOH in MeOH	[158]
WAX	PFC/ Fish, vegetables and soil	Ultrasound extract in H_2O pH 7	1) 1 mL 2% HCOOH, 2) 1 mL 95:5 H_2O:MeOH	4 mL 2.5% NH_4OH in acetone	[136]

Table 5. (Cont.).

Type sorbent	Analyte/ Matrix	Load Vol/pH	Wash Vol/solvent	Elution Vol/solvent	Ref
WAX	Flame retardants/ Urine	1 mL urine	1) 2 mL H_2O, 2) 2 mL 2% MeOH in H_2O	4 mL 2% tributylamine in MeOH	[170]
	Metabolites/ Urine	2 mL urine pH 2 (HCOOH)	2 mL H_2O	1) 2 mL 5% NH_4OH in MeOH, 2) 2 mL ethyl acetate	[167]

WW: Wastewater

From this table, it can be observed that some studies use protocols very similar to the recommended procedures, while other studies include some changes. The main difference between them is whether or not they include a washing step with a pure organic solvent. For example, when dealing with environmental samples, some authors describe the use of a few millilitres of pure MeOH to wash the sample, while others use aqueous solutions with small percentages of MeOH. Sometimes, the MeOH fraction is considered as elution if target analytes (with neutral properties or ionized with the same charges as the sorbent) are rinsed during this step. For instance, the method to determine herbicides in stormwater reported an elution procedure in two steps: firstly, MeOH to elute neutral and basic herbicides and, secondly, an acidic methanolic solution to elute acidic herbicides [137]. For biological samples, the use of pure organic solvent washing steps is present or absent depending on several factors and the use of solvents other than MeOH is also frequent. In the method developed for the determination of new psychoactive substances in body fluids the washing step included water, an acidic aqueous fraction, a mixture of CH_2Cl_2 and MeOH and hexane to clean up the sample [169]. The method for fungicides in wine samples used an aqueous washing step followed by elution in pure MeOH, because the idea of the protocol was to retain ionic interferences on the mixed-mode sorbent [173]. As mentioned before, polymeric exchangers can be used for several

purposes and the analytes of interest can be rinsed in the desired fraction by carefully selecting the SPE conditions.

The main limitation of mixed-mode polymeric sorbents is that they can have cation or anion-exchange properties, but they cannot have both. These sorbents are able to retain acidic and basic compounds simultaneously only if the washing step with pure organic solvent is not included, because the compounds retained only by reversed-phase interactions will not be rinsed. However, if MeOH is percolated during a washing step, the ions with the same charge as the sorbent will be rinsed from the sorbent. In many cases, the list of target analytes includes both acidic and basic compounds and the inclusion of washing steps to eliminate neutral compounds is also required.

To overcome this limitation, a number of studies have combined anion and cation-exchangers to retain acidic and basic compounds sequentially. Lavén *et al.* [184] proposed an SPE protocol for the extraction of 15 basic, neutral and acidic pharmaceuticals from wastewaters using Oasis MCX followed by Oasis MAX cartridges in the same procedure. A representation of the SPE protocol used by the authors is shown in Figure 6.

In this procedure, the fraction obtained from the first elution step (pure MeOH) in the Oasis MCX cartridge was diluted and loaded onto the Oasis MAX sorbent. For both cartridges, the recommended protocol was used, adding aqueous solutions for clean-up purposes, which were not collected. In the end, three fractions of less complexity were obtained containing the neutral, basic and acidic pharmaceuticals in separate solutions. The authors reported average recoveries of 80% and ME values around -30% using this SPE procedure. However, some analytes exhibited strong ion suppression (e.g. -85% was recorded for hydrochlorothiazide in influent samples) and, with this in mind, a quantitation method was proposed to compensate for the ME.

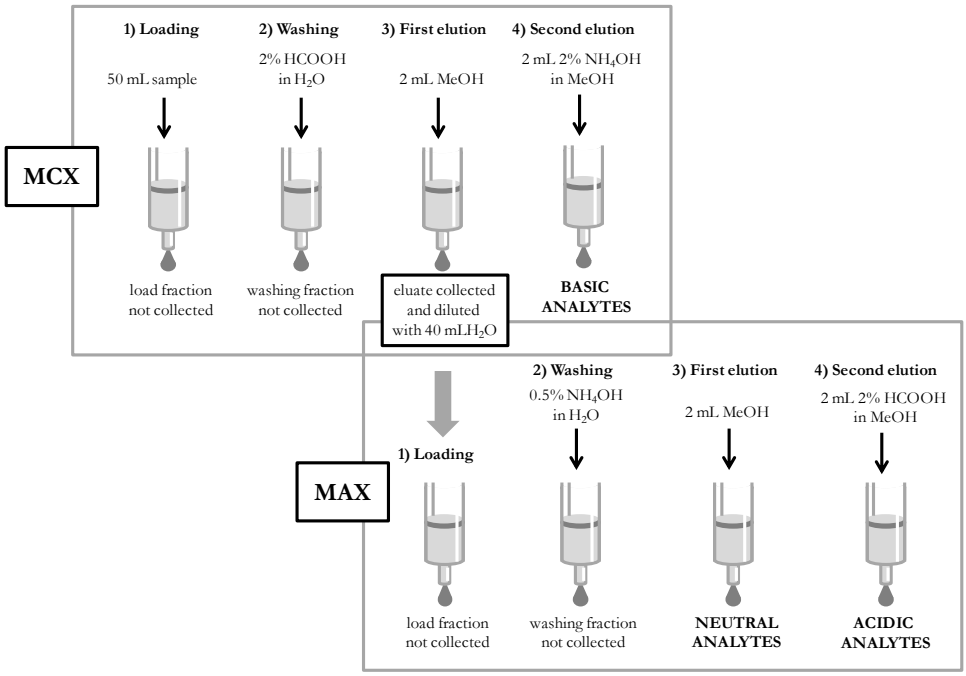

Figure 6. Full SPE protocol described in the study by Lavén *et al.* [184] to extract pharmaceuticals from wastewaters.

More recently, Deeb *et al.* [185] developed a method to extract several micropollutants from environmental waters based on the tandem coupling of Oasis MAX and Oasis MCX cartridges. In ultrapure water, results were better for this tandem combination than several other single cartridges (Oasis HLB, Oasis WAX, Oasis WCX, Strata-X, Strata-X-C, Strata-X-A) and other combinations (like Oasis HLB-MAX, Oasis HLB-MCX). The sample was loaded into the connected cartridges, after which they were disconnected, in order to perform the washing step and the elution separately from each other. Oasis MAX was washed with 2 mL of 5% NH_4OH in H_2O and eluted with 6 mL of 2% HCOOH in MeOH:ethyl acetate (69:29, v/v), whereas Oasis MCX was washed with 2 mL of 2% HCOOH in H_2O and eluted with 6 mL of 5% NH_4OH in MeOH/ethyl acetate (67.5:27.5, v/v). After the elution step, both eluates were combined for analysis in the LC-MS/MS instrument. Using this approach, recoveries were above or equal to 90% for

all of the analytes and the ME reported was low for all matrices. However, it can be observed how the selectivity of these sorbents was not fully exploited in this study, as a washing step with pure organic solvent was not included in the SPE protocol. The previously mentioned studies proposed configurations in which cation and anion-exchangers were combined to extract compounds with basic and acidic properties simultaneously. However, the protocols were more time-consuming than conventional SPE procedures.

Despite the introduction of HILIC and mixed-mode ion-exchange polymeric sorbents as recent alternative techniques for improving the determination of polar compounds, there is still demand for analytical methods applying these approaches for several polar analytes relevant to the environment, which exhibit certain limitations when analysed by conventional methods.

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

1.3. References

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Daniela Salas Acosta

CHAPTER 2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

The main objective of this Doctoral Thesis is to develop analytical methods to determine polar compounds in environmental waters, applying recent alternative techniques focused on improving aspects concerning their chromatographic separation and sample preparation.

This general aim is divided into two specific objectives: the first one is related to the application of hydrophilic interaction liquid chromatography studying its retention mechanisms and its advantages for the analysis of polar analytes in complex matrices; and the second, the development and evaluation of mixed-mode ion-exchange solid-phase extraction sorbents, proposing alternative and novel applications.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

As mentioned in the Introduction, several emerging organic contaminants (EOCs) present in environmental waters are compounds with polar properties, which have exhibited separation and retention problems when applying the chromatographic and extraction techniques conventionally used in modern analytical methods. To overcome these limitations, alternative techniques have been introduced such as hydrophilic interaction liquid chromatography (HILIC) or mixed-mode ion-exchange solid-phase extraction (SPE) sorbents, proving to be successful not only within the environmental field but also for biological and food applications. This Doctoral Thesis, as indicated before, focuses on the development of new selective and sensitive analytical methods applying these techniques to polar compounds relevant to the environment.

The present chapter contains the experimental part, results and discussion of the different studies developed during this Doctoral Thesis, which are classified in three sections. Each section includes a brief introduction to settle the background of the research, the results in journal paper format and the discussion of the most relevant results at the end of each section. These results have already been published or are in the progress of being published in different peer-reviewed scientific journals. In Appendix II, a list of the articles derived from this Doctoral Thesis is provided.

The first section is focused on the application of HILIC for the development of novel analytical methods to determine compounds with environmental relevance. In this sense, the HILIC separation is described for two groups of EOCs: iodinated X-ray contrast media (ICM) and artificial sweeteners. For the group of ICM, special attention was paid in studying the effect of the mobile phase parameters and type of stationary phase on chromatographic retention as well as the retention mechanisms driving the separation of these compounds. In the case of artificial sweeteners, a HILIC-HRMS method was developed and further validated in environmental waters, evaluating how these highly polar analytes can benefit from this LC mode in terms of chromatographic separation, enhanced retention and ionization in the HESI-HRMS instrument.

The second and third sections are focused on expanding the application of mixed-mode ion-exchange polymeric sorbents beyond their conventional uses. As recommended in typical extraction protocols, the studies of this section aimed to retain target analytes through ionic interactions, in order to include a washing step with pure organic solvent for the elimination of neutral interferences. However, the applications developed are not conventional because either the compounds studied were neutral under extraction conditions, or the sorbents were combined to obtain a single cartridge with amphoteric properties, for the simultaneous retention of basic and acidic compounds. Therefore, two novel analytical methods are reported for the extraction of two kinds of contaminants: a group of benzotriazoles, benzothiazoles and benzesulfonamides and a group of pharmaceuticals.

Motivated by the results obtained in the mentioned studies, the third section reports the synthesis and analytical evaluation of novel mixed-mode sorbents with zwitterionic moieties. These materials were developed during research stay in collaboration with Prof. Peter A. G. Cormack of the Polymer Research Group of the Department of Pure and Applied Chemistry of the University of Strathclyde (Glasgow, Scotland, United Kingdom).

The studies presented in this Doctoral Thesis are framed within one of the research lines of the group associated to projects of the Ministry of Economy and Competitiveness (CTQ2011–24179 and CTQ2014-52617-P) and the Department of Innovation, Universities and Enterprises (2009SGR223), focused on the development and application of new materials for sample treatment techniques for the determination of emerging organic contaminants. Also, financial support was given by the Ministry of Economy and Competitiveness through Professional Researcher Training (BES-2012-057792) and Research Stay Mobility (EEBB-I-15-10296) grants.

3.1. Hydrophilic interaction liquid chromatography as an alternative for the separation of polar compounds of environmental interest

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

For analytical chemists, the determination of organic compounds in the environment is very challenging because they are present at trace levels and environmental matrices are quite complex. In fact, new strategies are in continuous development to improve the selectivity and sensitivity of environmental analytical methods. Moreover, additional problems arise when the analytes have polar character, and this is the case of a significant number of the contaminants present in environmental waters [1].

Analytes with polar properties often exhibit lack of retention on the reversed-phase columns traditionally preferred in LC [2]. Several chromatographic strategies have been developed to address this limitation, giving rise to other LC techniques like ion chromatography or ion pair chromatography [3, 4], conceived for ionizable compounds. But none of these LC modes have been as successful and promising as hydrophilic interaction liquid chromatography (HILIC) in retaining and separating polar compounds and offering advantages when coupled with MS-based detectors. This superiority relies on the use of high organic content and aqueous mobile phases which improve the ionization and disolvation processes in the ion source. In addition, the development of a wide number of HILIC phases open several possibilities for obtaining separations of increased quality and selectivity [5].

As proved by several studies [5-7], a water layer is formed on the surface of the polar stationary phases used in HILIC, which allows the hydrophilic partition of the analytes between this layer and the mobile phase mostly organic. This retention mechanism is often present together with other interactions like adsorption, ion-exchange, hydrogen bonding or hydrophobic interactions, being HILIC defined by a multimodal separation mechanism. Several authors have dedicated a lot of effort in understanding HILIC mechanism because its complexity relies on being very dependent on the properties of the stationary phase, analytes and the mobile phase conditions. The study of the effect of these parameters on the retention of a particular group of analytes is therefore very important to predict retention times, select the most suitable stationary column or gain insights on the predominant mechanisms driving retention.

So far, several applications using HILIC has been reported in the environmental field, as it is shown in Section 1.1.3.1. However, there is still a need for novel analytical methods using this LC mode as the list of polar analytes with environmental interest is long and in continuous increase, because novel drugs are developed, unknown metabolites and transformation products are discovered or substances are newly recognized as contaminants. Furthermore, the results obtained for compounds with different properties and chemical structures contribute to the overall understanding of this promising LC mode.

The studies included in this section describe the HILIC separation of two groups of analytes with environmental interest. These studies were the first deep investigations of our research group using this LC mode, for which the first study was focused on understanding the influence of the mobile and stationary phase parameters on chromatographic retention, as well as the retention mechanisms involved in order to learn and predict this technology. In this sense, the separation of a group of iodinated X-ray contrast media was studied by testing different columns and mobile phase conditions. The contribution of hydrophilic partition and adsorption to the overall retention was evaluated together with temperature studies to gather information of the retention mechanisms present during separation.

Once familiarized with the HILIC mode and understood the mechanisms and parameters involved, we took one step further to study its coupling with HRMS detection, evaluating its advantages when dealing with complex matrices such as environmental waters. With this in mind, the second study is focused on development and validation of a HILIC-HRMS method for the determination of artificial sweeteners in environmental waters, taking into account the compatibility of HILIC with the elution conditions of the SPE and the reduction of the matrix effect. To the best of our knowledge, this is the first HILIC-HRMS method determining these compounds in environmental matrices.

The results derived from these HILIC applications were published in the *Journal of Separation Science* 37 (2014) 1111-1117 and *Analytical Bioanalytical Chemistry* 407 (2015) 4277-4285, and are presented in the following sections.

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

3.1.1. Study of the retention behavior of iodinated X-ray contrast agents in hydrophilic interaction liquid chromatography, comparing bare silica and zwitterionic stationary phases

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

STUDY OF THE RETENTION BEHAVIOR OF IODINATED X-RAY CONTRAST AGENTS IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY, COMPARING BARE SILICA AND ZWITTERIONIC STATIONARY PHASES

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Abstract

Iodinated X-ray contrast media are the most widely used pharmaceuticals for intravascular administration in X-ray diagnostic procedures. The increasing concern of the fate of these compounds into the environment has led to the development of analytical methods to determine them. However, these methods present problems due to the polar character of these analytes. In this paper, hydrophilic interaction liquid chromatography is presented as an alternative technique. The retention of iodinated X-ray contrast media was studied in two bare silica phases with different particle designs (i.e. porous and Fused Core™) and a zwitterionic sulfoalkylbetaine phase. The effect of the most important parameters of the mobile phase was studied for each stationary phase. It was observed that optimal mobile phase conditions included buffers with a high buffering capacity. Additionally, the retention mechanisms involved were studied in order to provide some insight into the possible occurring interactions. The contributions of partition and adsorption and the effect of the temperature on the retention of analytes were evaluated on all of the stationary phases.

Keywords: *hydrophilic interaction liquid chromatography; iodinated X-ray contrast media; retention mechanisms.*

1 Introduction

Iodinated X-ray contrast media (ICM) are polar pharmaceuticals for intravascular administration that are used in medicine to visualize organs and vessels in diagnostic procedures [1]. In recent years, ICM are considered emerging contaminants and analytical methods have been developed to determine them in environmental matrices. The potential impact of ICM has been considered since it was discovered that these compounds contribute substantially to adsorbable organically bound iodine (AOI) levels in hospital wastewaters [2]. In fact, ICM have been found in wastewaters, ground waters and even in drinking waters [3]. The reach of ICM into environmental waters is a consequence of the high dosages administered in each intake (60 to 120 g) and to their persistence and polar character [1].

Today, the methods used for the determination of ICM in environmental waters usually include solid-phase extraction (SPE) followed by RPLC separation and mass spectrometric detection. These methods have been validated and applied to real samples, but some troubles are described. Complex matrices usually cause high ionic suppression in LC-MS [2]. Also, high water proportions are needed in the mobile phase in order to favor retention of the analytes, making subsequent vaporization in the MS troublesome. In a previous study, 95% of water and a flow rate of 0.2 ml/min were needed in order to achieve suitable separations in three different RPLC stationary phases. Furthermore, it was found that the separation on a zwitterionic stationary phase using hydrophilic interaction liquid chromatography (HILIC) displayed better results compared to the RPLC separations [4].

HILIC has been developed as an alternative for the determination of polar and hydrophilic compounds that show poor retention in reversed phase liquid chromatography (RPLC). It is a promising technique that uses a polar stationary phase (as in NPLC) and the mobile phase is an organic/aqueous mixture (as in RPLC) but with a high organic content, that offers low backpressures and facilitates spraying and desolvation when MS detection is employed [5-9].

The selection of the stationary phase and mobile phase conditions is a very important step in the method development as a

great number of materials with different functional groups are available nowadays [6, 10]. Bare silica and zwitterionic sulfoalkylbetaine stationary phases are among the most frequently used materials in HILIC applications [10-12]. Analyte retention on a particular stationary phase depends on many factors and the understanding of the mechanisms involved can offer valuable information. In HILIC, analytes can undergo partition between a layer of water that is formed on the surface of the stationary phase and the mobile phase that would be mostly organic [11, 13]. However, along with partition, other retention mechanisms can take place, such as adsorption, ionic interactions, hydrogen bonding or even hydrophobic interactions [5, 6, 11]. Identifying which is the predominant interaction can be difficult because it depends on many factors. For this reason, the theory at the basis of HILIC mechanism is yet not fully understood and is still under investigation [14-16]. The relative contributions of partition and adsorption to the retention mechanism in HILIC have been studied by several authors [5, 6, 11, 17, 18], and also the relationship between retention and temperature [6, 17, 19, 20].

Considering that the chromatographic separation of ICM is particularly troublesome, the aim of this paper is to present HILIC as an alternative technique to separate them studying the effect of the most important parameters of the mobile phase in the retention on three different stationary phases: a bare silica porous particle stationary phase, a second bare silica phase but with a Fused Core™ particle technology and a zwitterionic porous particle stationary phase. Also, the retention behavior of the analytes on the stationary phases is discussed in order to provide some insight into the possible interactions occurring in the separation.

2 Experimental

2.1 Reagents and standards

Iopromide, iopamidol, iomeprol and diatrizoic acid were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and iohexol was supplied by Sigma-Aldrich (St. Louis, USA). Stock solutions of 1 mg/ml were prepared by dissolving each solid standard in methanol (MeOH) and were stored at -20°C. Working solutions of a mixture of all compounds were prepared in 1:3 MeOH:ACN (v/v) at a concentration of 5

mg/L and were stored at 4°C in the dark. The chemical structures, CAS number and LogP and pK_a values of the analytes are shown in Table S1 (electronic supplementary material).

Ultrapure water was obtained from a water purification system (Veolia, Sant Cugat del Vallès, Spain) and was used to prepare the mobile phases. HPLC grade MeOH and acetonitrile (ACN) were purchased from VWR (Llinars del Vallès, Spain) and Merck (Darmstadt, Germany), respectively. Analytical grade ammonium acetate (CH_3COONH_4) and ammonium formate ($HCOONH_4$) were supplied by Sigma-Aldrich. Acetic acid (CH_3COOH) from SDS (Peypin, France), formic acid (HCOOH) from Sigma-Aldrich and ammonium hydroxide (NH_4OH) from Panreac (Barcelona, Spain) were used to adjust the pH of the mobile phases. Each mobile phase tested was filtered through a 0.22 μm nylon filter (Scharlab, Barcelona, Spain) before use.

2.2 Instrumentation and columns

All experiments were performed using an Agilent Technologies 1100 series HPLC system, equipped with a quaternary pump, UV detector, a solvent degasser unit, a 20 μL loop manual injector and a column heater.

Three HILIC stationary phases were tested to evaluate the retention of the ICM. Two bare silica stationary phases with different particle designs were used: the fully porous particle column Atlantis HILIC Silica (100 mm x 2.1 mm i.d., 5 μm particle size) supplied by Waters (Milford, MA, USA) and the Fused-Core™ particle column Ascentis Express HILIC (50 mm x 2.1 mm i. d., 2.7 μm particle size) purchased from Supelco (Bellefonte, PA, USA). The zwitterionic phase ZIC-HILIC column (150 mm x 4.6 mm i.d., 5 μm particle size) supplied by Merck was also tested.

2.3 Chromatographic conditions

The mobile phases tested were mixtures of ACN and aqueous solutions at various pH levels and salt concentrations. The pH values and salt concentrations reported were the values measured or calculated for the aqueous fraction of the mobile phase, before mixing the organic solvent.

For both silica phases, the gradient profile started with 98% ACN and

was reduced to 90% in 10 minutes, which was then held for 5 minutes. For the zwitterionic stationary phase, the gradient profile started with 95% ACN and was reduced to 86% in 23 minutes. A period of 5 minutes was included in both profiles in order to return to the initial conditions, which were then held for 5 to 8 minutes to equilibrate the column for the subsequent analysis.

The optimal flow rate was 0.5 mL/min for the silica columns and 1.0 mL/min for the zwitterionic column. The temperature of the column oven was set at 25°C for silica columns and at 65°C for the zwitterionic column. The wavelength selected for the detection of all the ICM was 240 nm.

3 Results and discussion

3.1 Optimization of chromatographic conditions

Mobile phase parameters (e.g. organic solvent, mobile phase pH, salt concentration and type of salt) can have a significant impact on analyte retention in HILIC [6]. The most commonly used organic solvent is ACN as it gives better results in a great number of applications [10, 21] and it was used in this study. Ionic additives, such as ammonium acetate and formate, are commonly employed together with acetic and formic acid to control mobile phase pH and ionic strength considering the suitability of these salts for MS detection

This section describes the analyte retention behavior that was observed when pH, salt type and concentration were varied. The effect of these parameters was studied one at a time, keeping the rest of the conditions constant.

3.1.1 Porous particle bare silica stationary phase

For this stationary phase, a $HCOONH_4$/HCOOH buffer was tested at pH 3.75 with different salt concentrations (0, 10, 20, 50 and 100 mM) followed by 10 mM and 100 mM $HCOONH_4$ /HCOOH solutions both adjusted to pH 3 and 5. In addition, a 10 mM CH_3COONH_4/CH_3COOH buffer was tested at pH 3, 3.75 and 5, together with a 100 mM buffer adjusted to pH 3.75. These pH values were selected to comprise the feasible pH range with the stationary phase limitations and including the optimal pH values of the buffers.

Based on the results, the most significant observation was that

selectivity was strongly improved when a high concentration buffer was used, since complete peak separation was achieved. In contrast, low buffer concentration displayed coelution of the compounds. Moreover, the retention times of all of the analytes increased when the salt concentration of the buffer increased.

Silica stationary phases may exhibit partition or adsorption interactions thank to the hydrogen bonds that the silanol groups can establish or ionic exchange when they are deprotonated [10, 11]. Thus, higher retention at high salt concentrations may be explained by the enhancement of hydrogen-bonding interactions between the analyte and the silanol groups of the stationary phase, facilitated by salting-out effect. Water molecules prefer to solvate salt ions rather than analytes, leaving them free to interact with the stationary phase [10]. Another explanation of the retention improvement could involve an increase of the water layer thickness when the salt concentration rises [6, 17]. Therefore, it can be seen that the optimal buffer concentration was 100 mM.

Regarding to pH, the lowest retention was observed at pH 5 in most of the cases, with diatrizoic acid being the most affected analyte. The decrease in retention observed for diatrizoic acid (the only charged compound in the mixture) from pH 3 to pH 5 can be attributed to ionic repulsion between the analyte and the deprotonated silanols on the surface of the stationary phase, which may already be present above pH values of 4 to 5 [6]. The rest of the analytes are neutral, so ionic interactions cannot be present. However, at pH 5, less silanol groups are available because they are now deprotonated, probably resulting in a lower number of hydrogen bonds between the analytes and the silica material.

Between the two type of buffers, it was found that selectivity was slightly better and retention higher when the $HCOONH_4$/HCOOH buffer was employed, which proved to be the best option for mobile phase preparation. This $HCOONH_4$/HCOOH buffer showed the highest retention at pH 3.75, which can be attributed to the higher buffering capacity of this solution at this pH value. To summarize, the buffer selected as optimal was a 100mM $HCOONH_4$/HCOOH mixture at pH 3.75 because, under these conditions, all of the analytes were

satisfactorily separated (as shown in Figure 1A).

Using the previous buffer, flow rate and temperature were optimized comparing 0.3, 0.5, 0.8 and 1.0 mL/min and 25°C, 35°C and 45°C. A flow rate of 0.5 mL/min was chosen because it allowed the separation of analytes within 12 minutes, showing better separation for diatrizoic acid and iohexol in comparison to higher flow rates. High temperatures caused peak distortion, so 25°C was selected as the optimal temperature.

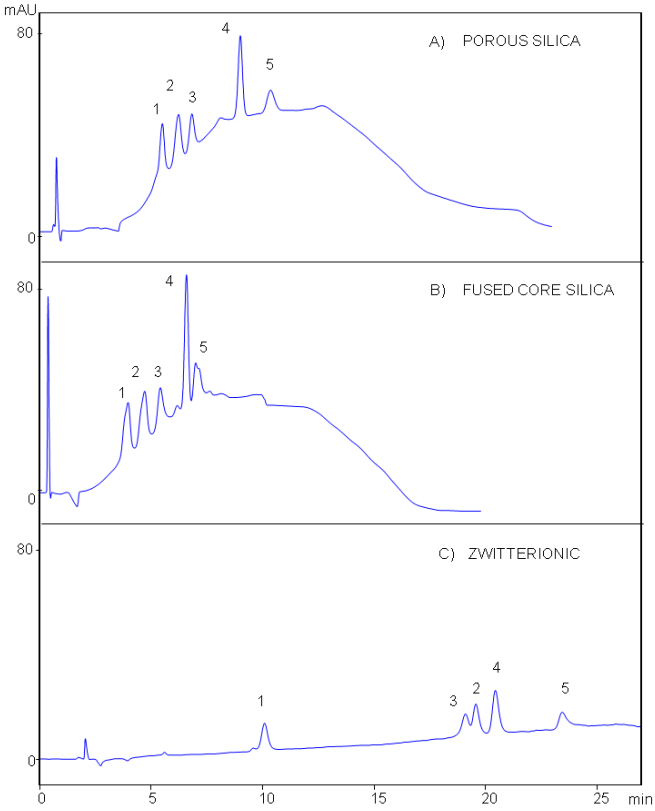

Figure 1. Chromatograms of ICM on A) porous particle bare silica, B) fused core particle bare silica and C) zwitterionic stationary phases at optimum mobile phase condition (silica, 100 mM $HCOONH_4$/HCOOH at pH=3.75; zwitterionic, $CH_3CHOONH_4$/CH_3COOH at pH=4.75). Peak identities: 1 iopromide, 2 iopamidol, 3 iomeprol, 4 diatrizoic acid, 5 iohexol.

3.1.2 Fused Core™ particle bare silica stationary phase

Subsequent to prior optimization, another bare silica phase with Fused Core™ particles was studied in order to improve efficiency and analysis time, which are the advantages attributed to this type of material. Based on previous results, a 100 mM $HCOONH_4$/HCOOH buffer was tested adjusted to pH 3, 3.75 and 5. When adjusted to pH 3.75, it was also tested at a salt concentration of 10 and 50 mM. A 100 mM CH_3COONH_4/ CH_3COOH buffer adjusted to pH 3.75 was then also tested.

It was realized that optimal conditions were exactly the same for both silica stationary phases, with the best option being the 100 mM $HCOONH_4$/HCOOH buffer adjusted to pH 3.75. As expected, based on previous observations, the selectivity of the chromatographic separation improved when salt concentration was increased. Retention times were longer at higher salt concentrations for all of the compounds with the exception of iomeprol. With respect to pH, little difference was observed between pH 3 and 3.75, while lower retention and coelution was observed at pH 5. Working at pH 3, a higher baseline drift was observed. This could be solved by adding buffer to the organic portion of the mobile phase. However, due to lack of salt solubility, it was decided to work at pH 3.75 which displayed good retention and less baseline distortion. For the optimization of the flow rate and temperature, the same values used for the previous phase were tested. At high flow rates, overlapping was observed, so 0.5 mL/min was selected, achieving total separation within less than 10 minutes. The optimal temperature selected was 25°C because higher temperatures caused overlapping and peak distortion.
In Figure 1B, it can be observed that a shorter column with Fused Core™ particles yielded faster analysis and satisfactory separation but peak shape and resolution between iohexol and diatrizoic acid was better for the porous phase.

3.1.3 Zwitterionic stationary phase

A totally different stationary phase, which has a sulfoalkybetaine group and displays great separation for a large number of polar

compounds, was studied testing 10 and 100 mM $HCOONH_4$ /HCOOH buffers, both adjusted to pH 3.75, and a 100 mM CH_3COONH_4/CH_3COOH buffer at pH 4.75. It was found that, changing neither pH nor salt conditions produced significant differences in selectivity and retention of the compounds. However, it was noticed that in order to achieve reproducibility of diatrizoic acid retention times, the aqueous phase must be under high buffering capacity conditions. As this compound is the only acid in the mixture, a different retention mechanism may be expected in comparison to the rest. This stationary phase contains a quaternary ammonium and a sulfonic acid group in the same ligand, separated by a short alkyl chain. Thus, the surface of the material has only a low net negative charge due to the surface sulfonic groups [10, 12, 22]. Therefore, the interaction affecting diatrizoic acid may be ionic. Thus, both 100 mM buffers adjusted to high buffering capacity pH values (3.75 and 4.75) are appropriate for ICM separation with this stationary phase. However, baseline distortion was significant when the $HCOONH_4$ /HCOOH buffer was tested, so the CH_3COONH_4/ CH_3COOH buffer was selected as optimal (Figure 1C). Once the buffer conditions were optimized, the temperature and flow rate were evaluated with 0.3, 0.5, 0.8 and 1.0 mL/min being tested, at temperatures of 25°C, 35°C, 45°C, 55°C and 65°C. Low flow rates caused peak overlapping, so 1.0 mL/min was chosen. The highest temperature (65°C) was also selected because iomeprol and iopamidol were separated under these conditions. Selectivity changes in zwitterionic materials with increasing temperature have been attributed to the increasing flexibility of the intercharge chain of the stationary phase which can favor formation of an internal salt, leading to a reduction in ionic interactions [23].

Selectivity was different between silica and zwitterionic phases but elution order was quite similar. It should be noted that the gradient required for the elution of the analytes was smoother for the zwitterionic phase than for silica. As expected, the elution order observed in this work was different from RPLC methods [1], however it was not exactly opposite. Iohexol, which is poorly retained with RPLC, presented the highest retention in all HILIC phases. Iopromide, which is highly retained with RPLC, eluted first in our studies. However, the rest of the analytes presented similar

retention in both chromatographic modes, especially in the case of iopamidol, which was first or second to elute in both RPLC and HILIC.

Analysis time was higher for the zwitterionic material, being twice the time of that obtained for both silica stationary phases. In addition, pH, salt type and salt concentration were found to have little influence on retention. Moreover, the resolution between iomeprol and iopamidol was poor in comparison to the fully chromatographic separation obtained for silica phases. For these reasons, silica stationary phases were selected as the best option. Between the fully porous and Fused Core™ particle materials, better peak shape was observed when using the porous particle phase.

3.2 Relative contribution of partition and adsorption to the retention mechanism

Once the separation conditions had been optimized in the previous stationary phases, the mechanisms involved between the analytes and each phase were studied. To do so, the relationship between retention and aqueous proportion in the mobile phase was evaluated by constructing plots of log k vs. volume fraction of water (φ) and log k vs. log φ. Data was collected from the injection of individual standard solutions of all analytes at different isocratic elutions, covering an aqueous percentage range from 5% to 25%. Mobile phase conditions were the same as those previously optimized but they were adapted to maintain the salt concentration constant by adding salt to ACN. For bare silica phases, it consisted of solution A: water/ACN (5/95) % containing 10 mM of $HCOONH_4$ and solution B: 10 mM $HCOONH_4$/HCOOH buffer at pH 3.75. In the case of the zwitterionic phase, the mobile phase consisted of solution A: water/ACN (5/95) % containing 10 mM of CH_3COONH_4 and solution B: 10 mM CH_3COONH_4/CH_3COOH buffer at pH 4.75. For all of the stationary phases, the analytes eluted at void time at buffer proportions higher than 30%.

In the case of the fully porous silica phase, curvature was observed in the log-linear plot showing unsatisfactory determination coefficients ($0.9346 < R^2 < 0.9746$), while linearity was improved for the log-log plot ($0.9920 < R^2 < 0.9982$) (Figure 2A and 2B). Plots made of k vs. the linear and logarithmic function of

the water proportion should yield straight lines for partition and adsorption interactions respectively, indicating which retention mechanism is predominant [5, 11]. Therefore, linearity of plots obtained for the porous particle bare silica stationary phase favored the adsorption process. Similar results were obtained for the Fused Core™ particle stationary column, which was expected as they have the same chemistry. Despite the retention of water on silica favoring partition, adsorption seems to be predominant in the separation of the ICM. It has been proved that molecules with hydrogen-donor functionalities can interact through the hydrogen bond with the stationary phase and the importance of these interactions in HILIC has been confirmed [8, 14].

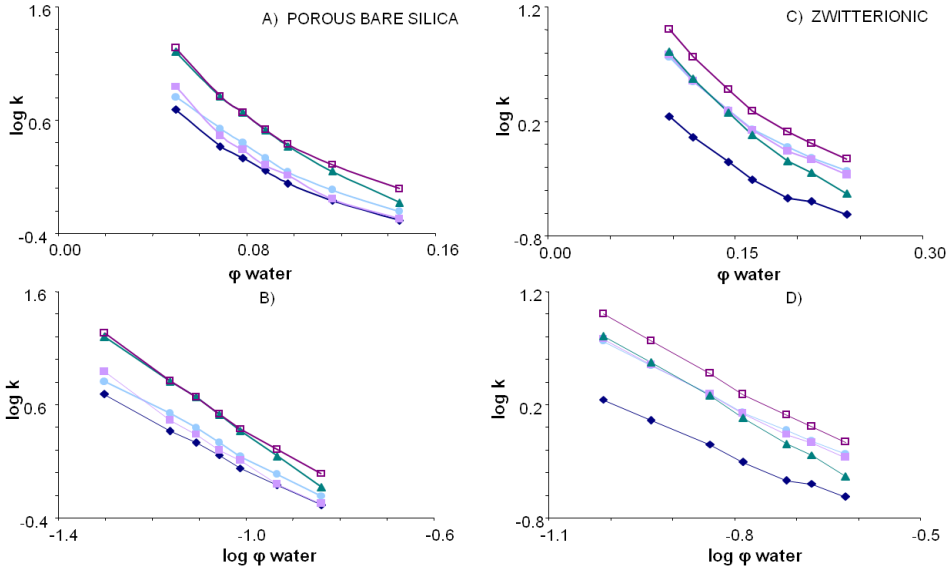

Figure 2. Plots of log *k* vs. volume fraction of water in the mobile phase and vs. log volume fraction of water obtained for the porous particle bare silica stationary phase (A and B) and for the zwitterionic stationary phase (C and D). Analyte identities: ◆ iopromide, ■ iopamidol, ● iomeprol, ▲ diatrizoic acid, □ iohexol.

Iodinated X-ray contrast media are compounds with a high number of hydroxyl groups in their structure (Table S1), favoring hydrogen bonds between the analytes and the silanol groups of the stationary phase. Lower retention at pH 5 where silanols are deprotonated and the low quantity of water that is needed to elute the analytes (in HILIC adsorption processes may be more significant at low water concentrations when the layer of water is quite thin [14]) support the idea of a direct interaction with the stationary phase. This is also consistent with the retention behavior of these compounds, since the ICM with more hydroxyl groups were retained more than those with lower number of –OH groups (e.g. iohexol, which has six hydroxyl groups, was always retained more). Elution order can also be related to hydrophilicity as analytes with higher log P values eluted first than those with lower log P values. It can be seen how hydrophilicity and hydrogen bonding capacity correlate with each other for these compounds [24].

Plots obtained for the zwitterionic stationary phase showed similar behavior (Figure 2C and 2D). Linearity of the log-log plots improved ($0.9960 < R^2 < 0.9996$) when compared to log-linear plots ($0.9620<R^2<0.9826$), as indicated by the determination coefficients. This behavior suggests that adsorption may also be the predominant interaction for this stationary phase. This is surprising because zwitterionic materials strongly adsorb water [6, 11], so a mechanism based on partition was expected. However, it has been found that sulfobetaine ligands in the zwitterionic material have hydrogen acceptor functionalities and adsorption-like interactions with hydrogen donor analytes might occur. It should be taken into account that even when in our case data was better adjusted to an adsorption process, in several studies of other analytes data do not fit to any model.

Unequivocally identify the prevailing interaction can be difficult, and the theory at the basis of HILIC mechanism is still under investigation. For this reasons, other observations and experiments must be considered. Both equations previously studied are still very useful during the development of a chromatographic separation and for the characterization of new stationary phases [14].

3.3 Effect of column temperature on retention

In order to obtain more information on the mechanism that controls ICM retention, plots of In *k* vs. 1/T were constructed for these compounds in each stationary phase. Data was collected from the injection of individual standard solutions of all of the analytes at different column temperatures from 25ºC to 65°C (or the maximum temperature recommended depending on stationary phase stability). For silica materials, 100 mM $HCOONH_4$/HCOOH buffer at pH 3.75 was used along with isocratic elution with the mobile phase composition of 95% ACN/5% buffer (v/v). In the case of the zwitterionic phase, the mobile phase employed was 87% ACN/13% 100 mM CH_3COONH_4/CH_3COOH buffer (v/v) at pH 4.75. These mobile phase proportions were selected taking into account that both most and least retained compounds elute at adequate retention times.

Ln *k* vs. 1/T plots obtained for the porous particle silica and zwitterionic phases are shown in Figure 3. As expected, the higher the temperature the lower the retention of all analytes in all of the stationary phases. No pronounced curvatures were observed in the plots but linearity was not sufficient to obtain good squared determination coefficients ($0.8477<R^2<0.9979$). Only iomeprol, iohexol and iopamidol presented satisfactory determination coefficients ($0.9951<R^2<0.9979$) when separated in the Fused Core™ particle phase.

In theory, the Van't Hoff equation should model the retention behavior in HILIC [6, 20] when only hydrophilic partition is present between the adsorbed water layer and the mobile phase. Curvatures in Van't Hoff plots are an indication of a change in the predominant retention mechanism or the presence of multiple interactions [6, 10]. Thus, deviation of linearity could be attributed to the contribution of various mechanisms, but this does not support previous observations in relation to the retention vs. water proportion plots where adsorption seems to act as the predominant mechanism. Therefore, this could be an indication that partition might be involved as a contributing interaction as the Van't Hoff plots obtained were not linear. The idea of a multimodal mechanism where adsorption and partition are both present is more likely especially for the zwitterionic phase which easily

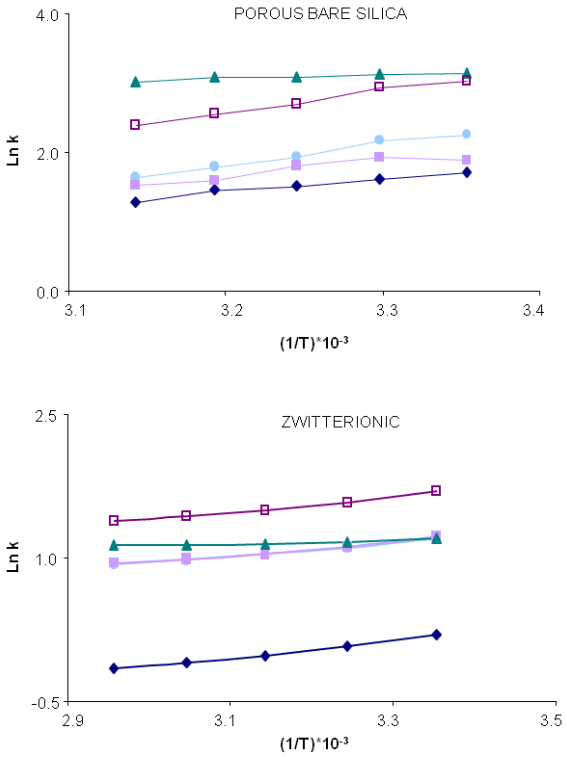

Figure 3. Van't Hoff plots obtained for the porous particle bare silica and zwitterionic stationary phases. Analyte identities as in Figure 2.

absorbs water on its surface. Interestingly, diatrizoic acid presented the lowest determination coefficients in comparison with the other neutral analytes. Apparently, this compound was influenced by additional interactions that did not affect the rest, which were probably electrostatic interactions between the negative charges of both analyte and stationary phase. However, it must be considered that deviation in Van't Hoff plots can be also explained by changes in the phase ratio of the stationary phase (Φ) which, in theory, must remain constant [20, 25]. In HILIC, differences in the phase ratio may be due to changes in the water layer thickness. As mentioned earlier, the highest determination coefficients for most of the analytes were

obtained when the Fused Core™ particle material was employed. This may indicate that differences in particle design have influence the phase ratio homogeneity.

Lastly, based on the slopes of the trend lines obtained for the Van't Hoff plots, approximate retention enthalpies (ΔH°) were calculated for all of the analytes in each stationary phase. All of the compounds showed negative enthalpies (-1.4 kJ mol^{-1} to -29.40 kJ mol^{-1}), indicating an exothermic process in all cases. Diatrizoic acid presented higher enthalpies compared with the rest of the analytes, indicating that more energy is necessary for transferring the analyte from the mobile phase to the stationary phase. This may be attributed to the repulsive character of the electrostatic interaction involved between diatrizoic acid and both types of stationary phases.

4 Concluding remarks

The retention behavior of ICM in HILIC was studied and it was found that this technique is a feasible alternative for the determination of these polar compounds. For all of the stationary phases, the optimal conditions involved mobile phases with buffers with high buffering capacity conditions. Selectivity and retention on bare silica stationary phases improved when high salt concentrations were employed. In the case of the zwitterionic phase, changes in chromatographic separation were not significant when pH or salt conditions were varied. The stationary phase that showed the best performance was the porous particle bare silica phase, as better selectivity and efficiency were achieved within a reasonable analysis time.

Plots to determine the relative importance of the partition and adsorption mechanisms to retention showed that adsorption may be predominant in all of the stationary phases studied. This is consistent with the capability of ICM to form hydrogen bonds with the stationary phases. However, temperature studies suggested that various mechanisms actually contribute to the overall retention, indicating that partition cannot be ruled out as a contributing factor. However, the curvatures of Van't Hoff plots can be also attributed to phase ratio changes. Therefore, whether or not adsorption is predominant remained inconclusive. It was presumed that ionic interactions were present for diatrizoic acid, the only ionic compound in the mixture.

Acknowledgments

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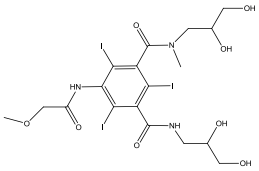
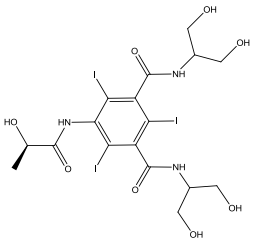
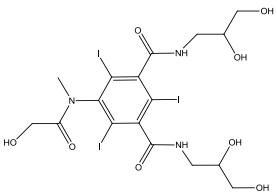
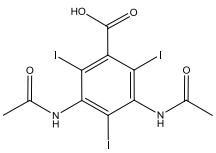
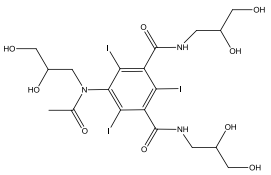
Supplementary Data

Table S1. Chemical structure, CAS number, Log P and pK_a values of the studied analytes.

Analyte	Structure	CAS number	Log P[a]	pKa[b]
Iopromide		73334-07-3	-2.1	6.6 12.1
Iopamidol		60166-93-0	-2.4	6.9 11.5 12.7
Iomeprol		78649-41-9	-2.3	10.6 11.8
Diatrizoic acid		117-96-4	1.8	1.1 8.5 11.0
Iohexol		66108-95-0	-3.0	10.6 11.8 14.0

[a] Values obtained from PubChem Compound Database (pubchem.ncbi.nlm.nih.gov)
[b] Values calculated using Sparc (http://archemcalc.com/sparc)

3.1.2. Hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry to determine artificial sweeteners in environmental waters

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY TO DETERMINE ARTIFICIAL SWEETENERS IN ENVIRONMENTAL WATERS

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Abstract

Artificial sweeteners are food additives employed as sugar substitutes which are now considered to be emerging organic contaminants. In the present study a method is developed for the determination of a group of artificial sweeteners in environmental waters. Considering the polar and hydrophilic character of these compounds, hydrophilic interaction liquid chromatography is proposed for their separation as an alternative to traditional reversed-phase liquid chromatography. Two stationary phases with different chemistry were compared for this purpose. For the detection of the analytes, high resolution mass spectrometry (Orbitrap) was employed to take advantage of its benefits in terms of reliable quantification and confirmation for the measurement of accurate masses. Solid-phase extraction was chosen as the sample treatment, in which the extract in a mixture of NH_4OH:MeOH:ACN (1:4:15) was directly injected into the chromatographic system, simplifying the analytical procedure. The optimized method was validated on river and waste water samples. For example, in the case of effluent water samples, limits of detection ranged from 0.002 to 0.7 μg/L and limits of quantification ranged from 0.004 to 1.5 μg/L. Apparent (whole method) recoveries ranged from 57% to 74% with intra-day precision (%RSD, n=5) ranging from 6% to 25%. The method was successfully applied to water samples from different rivers in Catalonia and different waste water treatment plants in Tarragona. Acesulfame, cyclamate, saccharine and sucralose were found in several samples.

Keywords: *artificial sweeteners; hydrophilic interaction liquid chromatography; high resolution mass spectrometry; environmental waters.*

1 Introduction

Artificial sweeteners are a class of food additives used as sugar substitutes in food, beverages, sanitary products and pharmaceuticals [1]. Among the list of artificial sweeteners (which comprise various classes of polar compounds), those approved by the European Union (EU) are acesulfame, aspartame, cyclamate, saccharin, sucralose and neohesperidin dihydrochalcone [2]. Moreover, stevioside and glycyrrhizic acid are sweeteners of natural origin of which consumption has recently increased [3,4]. The use of steviol glycosides as sweeteners has only been permitted in the EU since 2011 [3].

The use of these additives rather than sugar is preferred because they do not provide calories, their sweetness is quite intense, they do not cause blood glucose levels to rise and are also tooth-friendly. The consumption of artificial sweeteners can help to control obesity and diabetes but their use is often controversial due to suspicions of adverse health effects [1,2,5]. Therefore, to prevent potential risk to human health, regulations have been developed for these compounds, setting an upper limit on their concentration in different products, while some of them are even banned [2]. For example, cyclamate and neohesperidine dihydrochalcone are not included on the list of artificial sweeteners allowed in the USA [1,6]. Furthermore, recent studies have documented their widespread occurrence in the aquatic environment, becoming a new class of emerging organic contaminants (EOCs) in water samples. For these reasons, there is a need for accurate and reliable analytical methods to determine sweeteners not only in food samples, but also in environmental samples [7].

A large number of the existing analytical methods for the determination of artificial sweeteners have been developed for foodstuffs and beverages. However, the sensitivity of these methods is not suitable for environmental samples [2]. Considering that the list of artificial sweeteners that are employed in the industry is long, and some of the compounds are mixed to provide enhanced sweetness or improved taste, the simultaneous determination of several sweeteners in environment water samples is necessary [5,8]. The first liquid chromatography-tandem mass spectrometry (LC-

MS/MS) method to determine a group of seven sweeteners in environmental water samples was presented by Scheurer et al. [1,5]. Since then, the number of methods developed has increased but the need for validated multi-analyte methods for artificial sweeteners is still unaddressed [8].

The most common approach employed for determining these compounds is LC-MS/MS either directly injecting the sample or after a solid-phase extraction (SPE) procedure [2,5]. To the best of our knowledge, there are only two methods in the literature that employ hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS using QqQ as an analyser, and only one of these has actually been validated and applied to environmental water samples [5]. HILIC has been employed for polar and hydrophilic compounds due to the advantages that it offers. This chromatographic mode uses polar stationary phases (providing alternative selectivity to traditional reversed-phase LC) and mobile phases are aqueous/organic mixtures, in which the organic solvent is in a higher proportion. The high organic content of the mobile phase favors its coupling with MS detection because it facilitates spraying and desolvation in the interface, offers low backpressures in the LC system and enables the direct injection of organic extracts. The number of studies that have employed HRMS is lower compared to MS/MS, and most of these studies focus only on determining sucralose [9-13]. Using HRMS instruments, accurate masses from both molecular ions and fragments are observed, which is an advantage in complex matrices as a more accurate identification of the target analytes is possible [14].

In this work, the development and validation of a SPE/HILIC-HRMS method is described to determine a large group of commonly used sweeteners in environmental waters. To the best of our knowledge, this is the first HILIC-HRMS method developed for this group of analytes. HILIC conditions are optimized by testing two different stationary phases and different parameters affecting chromatographic separation of the analytes. In addition, SPE conditions are optimized to enable the direct injection of the organic extract into the HILIC system. Finally, the validated method is applied to environmental water samples.

2 Experimental

2.1 Reagents and standards

Potassium acesulfame (ACE), sodium cyclamate (CYC), saccharin (SAC), aspartame (ASP), sucralose (SUC), neohesperidine dihydrochalcone (NHDC), stevioside (STE) and glycyrrhizic acid (GLY) ammonium salt were purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of 1 mg/mL were prepared by dissolving each solid standard in methanol (MeOH) and were stored at -20°C. Only in the case of stevioside, a proportion of water (1:10 H_2O/MeOH) is needed to completely dissolve the solid. Working solutions of a mixture of all compounds were prepared in acetonitrile (ACN) and were stored at 4°C in the dark. The chemical structures, CAS number and LogP and pK_a values of the analytes are shown in Table 1.

Ultrapure water was provided by a water purification system (Veolia, Sant Cugat del Vallès, Spain) and HPLC grade MeOH and ACN were purchased from J. T. Baker (Deventer, the Netherlands). Analytical grade ammonium acetate (CH_3COONH_4) and ammonium formate ($HCOONH_4$) were supplied by Sigma-Aldrich. Acetic acid (CH_3COOH) from SDS (Peypin, France), formic acid (HCOOH) from Sigma-Aldrich, hydrochloric acid (HCl) from Scharlab (Barcelona, Spain) and ammonium hydroxide (NH_4OH) from Panreac (Barcelona, Spain) were used to adjust the pH of the mobile phase and the samples. Mobile phase was filtered through a 0.22 μm nylon filter (Scharlab) before use.

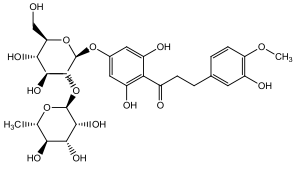
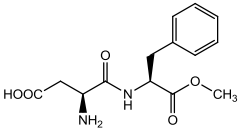
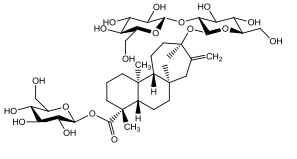
2.2 Sampling

Waste waters were collected from three urban treatment plants located in Tarragona, Reus and Vila-seca, all of which are in the Tarragona region in NE Spain. Influent and effluent samples were collected from Tarragona and Reus sewage treatment plants (STPs), which operate with a primary and a secondary treatment. Vila-seca STP operates with an additional tertiary treatment (a reverse osmosis process) and so influent and effluent samples from this tertiary treatment were also collected. River samples were collected from the Ebre, Segre, Ter, Llobregat and Francolí rivers. Water samples were collected in pre-cleaned bottles and were then stored at -20ºC. Prior to analysis, the samples were filtered through a 0.45 μm glass-fibre filter (Fisherbrand, Loughborough, UK). The pH of

Table 1. Chemical structure, pka, Log P, and exact masses of the studied analytes

Compound	Formula	CAS number	Structure	pKa[a]	log P[b]	Molecular ion (m/z)	Fragment (m/z)
Acesulfame (ACE)	$C_4H_4NO_4S$	55589-62-3		-0.28	-0.320	161.9866 $[M]^-$	82.0298 $[M\text{-}SO_3]^-$
Saccharine (SAC)	$C_7H_4NO_3S$	128-44-9		1.60	0.460	181.9917 $[M]^-$	61.9884 $[M\text{-}C_7H_5S]^-$
Cyclamate (CYC)	$C_6H_{12}NO_3S$	139-05-9		-8.66	1.033	178.0543 $[M]^-$	79.9574 $[M\text{-}C_6H_{12}N]^-$
Sucralose (SUC)	$C_{12}H_{19}Cl_3O_8$	56038-13-2		12.52	0.680	395.0073 $[M\text{-}H]^-$	359.0306 $[M\text{-}H\text{-}HCl]^-$

Table 1. (Cont.).

Compound	Formula	CAS number	Structure	pK_a[a]	log P[b]	Molecular ion (m/z)	Fragment (m/z)
Neo-hesperidine (NHDC)	$C_{28}H_{36}O_{15}$	20702-77-6		6.85	0.205	611.1981 $[M-H]^-$	303.0874 $[M-H-C_{12}H_{21}O_9]^-$
Aspartame (ASP)	$C_{14}H_{18}N_2O_5$	22839-47-0		3.71	1.110	293.1143 $[M-H]^-$	200.0717 $[M-H-C_2H_7NO_3]^-$
Stevioside (STE)	$C_{38}H_{60}O_{18}$	57817-89-7		12.51	1.19	849.3762 $[M-HCOO]^-$	641.3179 $[M-H-C_6H_{10}O_5]^-$

[a] Values obtained from Scifinder database.
[b] Predicted data using the ACD/Labs' ACD/PhysChem Suite (Chemspider database).

the samples was adjusted to 3 with HCl before the SPE procedure.

2.3 Solid-phase extraction

The extraction procedure was adapted from a previous study [15]. Oasis HLB cartridges (500 mg) supplied by Waters (Milford, MA, USA) were employed. STP water samples (50 mL for influent waters and 100 mL for both secondary and tertiary effluent and river samples) adjusted to pH 3 were loaded. A washing step with 10 mL of H_2O (pH = 3) was included in order to remove salts and highly polar compounds, followed by drying under vacuum. The analytes were eluted with 5 mL of a mixture of NH_4OH :MeOH:ACN (1:4:15). The extract was filtered with a 0.22 μm PTFE syringe filter and directly injected into the HILIC-HRMS instrument.

2.4 Hydrophilic interaction LC-high resolution mass spectrometry (HILIC-HRMS)

All experiments were performed using an Accela 1250 UHPLC system from Thermo Scientific (Bremen, Germany), equipped with a quaternary pump (1,250 bar), an Accela Autosampler automatic injector and a column oven. The LC system was connected by an Exactive Orbitrap™ mass spectrometer from Thermo Scientific. The interface employed was a heated electrospray ionization (HESI) source, operating in negative ionization mode. The instrument was equipped with a HCD collision cell in order to fragment the analytes for confirmation purposes.

The bare silica stationary phase Atlantis HILIC Silica (100 mm x 2.1 mm i.d., 5 μm particle size) supplied by Waters and the zwitterionic sulfoalkylbetaine phase Syncronis (100 mm x 2.1 mm i.d., 5 μm particle size) supplied by Thermo Scientific were tested to evaluate the retention of the sweeteners. The optimal mobile phase was a mixture of solvent A (100 mM $HCOONH_4$/HCOOH buffer adjusted to pH 3.75) and solvent B (ACN), for both silica and zwitterionic stationary phases. The gradient profile started with 98% ACN and was held for 2 minutes, before being reduced to 90% in 3 minutes, and subsequently reduced to 70% in 3 minutes and held for 4 minutes. A period of 5 minutes was included in the profile in order to return to the initial conditions, which were then held for 8 minutes to equilibrate the column for the subsequent

analysis. The optimal flow rate was 0.5 mL/min and temperature of the column oven was set at 25°C.

To optimize the HRMS conditions, a solution of each compound was introduced into the source by direct infusion (via syringe pump) together with a flow of mobile phase with 70% of ACN through a T connection. The signal of the negative molecular ions $[M]^-$ or $[M-H]^-$ of the analytes was monitored to optimize interface conditions in order to obtain the highest response for all of the compounds. Conditions were optimized in full scan at high resolution (50,000 FWHM) over a mass range of 100 to 1,000 m/z. The optimal parameters were: spray voltage, 3.5 kV; sheath gas, 40 AU (adimensional units); auxiliary gas, 5 AU; skimmer voltage, -26 V; capillary voltage, -25 V; tube lens voltage, -90 V; heater temperature, 350°C; capillary temperature, 300°C; and probe position adjustment: side to side, 0, vertical C and micrometer, 0.75. Two windows were used in negative mode with different collision voltages for fragmentation. Ions from characteristic fragments (Table 1) of the analytes were selected for confirmation and the HCD cell voltage was optimized to obtain the highest response for the fragment. The first window (0 to 7 min) used a full scan at 50,000 FWHM with 250 ms of injection time over a mass range of 100 to 1,000 m/z and a fragmentation scan at 10,000 FWHM with 50 ms of injection time over a mass range of 60 to 900 m/z at 20 eV in the HCD cell. The second window (7 to 25 min) used the previous scan events plus a third scan of fragmentation at 40 eV in the HCD cell using the previous fragmentation parameters. The molecular ions were measured for quantification (with a mass extraction window of 5 ppm) and fragments and the corresponding ion ratios were used for confirmation purposes. All of this information is shown in Table 1.

3 Results and discussion

3.1 Optimization of HILIC-HRMS conditions

The HILIC columns tested were a bare silica stationary phase and a zwitterionic material which has a quaternary amine and a sulfonic acid group in the same ligand, separated by a short alkyl chain. These two columns were tested because they have different chemistry and are commonly used in HILIC applications. For both stationary phases, a $HCOONH_4$/HCOOH buffer

adjusted to pH 3.75 and a CH_3COONH_4/CH_3COOH buffer adjusted to pH 4.75 were tested at different salt concentrations (10, 50 and 100 mM). Additionally, a 100 mM $HCOONH_4/HCOOH$ solution adjusted to pH 4.75 and a 100 mM CH_3COONH_4/CH_3COOH buffer adjusted to pH 3.75 were also assayed. In the case of the bare silica phase, a strong distortion and broadening of the peak corresponding to NHDC was observed for all mobile phase conditions, except for the 100 mM $HCOONH_4/HCOOH$ buffer. Under these conditions, higher retention of all the analytes was observed in comparison with lower concentrations of the same buffer, except for ASP. Furthermore, higher retention of all the analytes and best peak shape for NHDC was observed for the 100 mM $HCOONH_4/HCOOH$ buffer adjusted to pH 3.75 compared to the same buffer adjusted to pH 4.75. However, for all of the conditions tested, ACE and SAC were poorly retained and separation between STE and ASP was not satisfactory enough.

Meanwhile, when the zwitterionic sulfoalkylbetaine stationary phase was tested with buffers at high concentrations, peak shape and retention of the analytes were better. The optimal pH value for 100 mM buffers was that which had the highest buffering capacity for each buffer. When comparing buffer type, higher retention and better peak shape were observed for $HCOONH_4/HCOOH$ solutions. For the reasons stated above, a 100 mM $HCOONH_4/HCOOH$ buffer adjusted to pH 3.75 was selected as optimal. Applying these mobile phase conditions, flow rate and temperature were optimized by comparing 0.3, 0.5, 0.8 and 1.0 mL/min and 25, 35, 45 and 55°C. A flow of 0.5 mL/min was selected because, at higher flow rates, ACE and CYC overlapped and ASP showed peak distortion, while, at 0.3 mL/min, analysis time was longer. The optimal temperature was 25°C because higher temperatures caused overlapping and peak distortion. A chromatogram of the final separation is shown in Figure 1.

In general, retention was higher in the zwitterionic stationary phase compared to the silica material. Better separation between ASP and STE was achieved. Surprisingly, the elution order was practically the same, which might indicate that water layer conditions are more important than direct interaction between the analytes and the phase with respect to

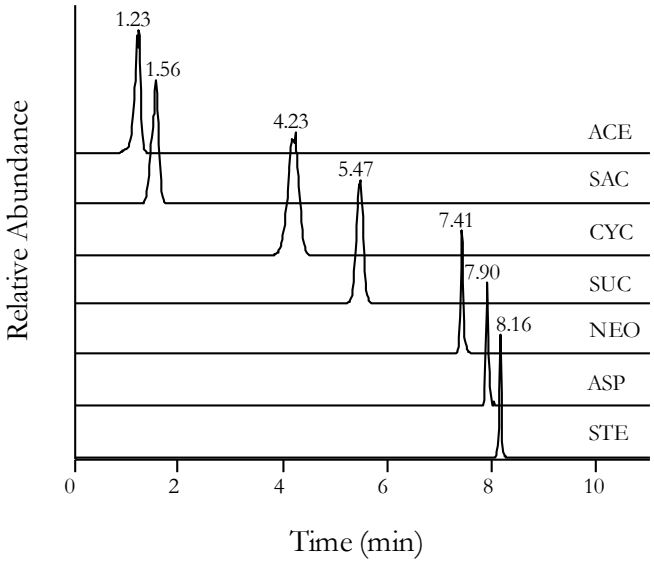


Figure 1. Chromatogram of the separation of a mixture of the analytes under optimal conditions.

retention. Better results were obtained compared to a previous study in which the HILIC column employed (the solid core bare silica Ascentis Express HILIC) did not give greater separation than that observed for the RP stationary phases evaluated, because four of the compounds eluted at void volume for the mobile phase conditions employed [15]. When comparing the present separation with the RP separation obtained in that study, a shorter analysis time was observed for the HILIC approach. Optimal HRMS parameters (described in Section 2.4) were chosen as a compromise between the highest responses obtained for each analyte. Ranges tested for each parameter were: spray voltage, 2 to 5 kV; sheath gas, 20 to 80 AU; auxiliary gas, 0 to 40 AU; skimmer voltage, -5 to -50 V; capillary voltage, 10 to 100 V; tube lens voltage, -50 to -200 V; heater and capillary temperature, 200 to 400°C and probe position adjustment including side to side position, -1 to +1, vertical, C or D and micrometer, 1 to 2. The range of HCD cell collision voltage tested was 5 to 70 eV. With respect to optics, tube lens voltage was the parameter that affected the response of the analytes most differently and for which the selection of a compromise was

hardest. For the gases flow rate optimization, mobile phase flow rate requirements were considered together with the effect on the response, which was similar for all of the compounds.

The entire acquisition was carried out in negative mode because negative molecular ions $[M]^-$ or $[M-H]^-$ were selected for quantification. STE displayed a formate adduct $[M+HCOO]^-$, as $HCOONH_4$ was added to the mobile phase. For this compound, the adduct was selected for quantification as its response was more abundant than $[M-H]^-$.

With respect to fragmentation, some fragments were clearly observed at low collision voltages (10 to 20 eV), while others were present at higher energies (>35 eV), as is the case for STE and NHDC. For this reason, two scans were selected at two different collision voltages (20 and 40 eV), which were selected as a compromise between the highest responses of all the fragments present at each voltage. One fragment was selected for each analyte, which results in at least 4 identification points, fulfilling European guidelines for residues [16]. Details of the selected fragments are shown in Table 1. For ACE, CYC and SAC, sulfur fragments (SO_3 for example) were selected. Even when these fragments are less characteristic, the response obtained was high, enabling distinction from noise. For NHDC and STE, more characteristic fragments were observed because of their more fragmentable structure. The fragment selected for STE might be the result of losing one of the six atom rings substituted with four hydroxyl groups. In the case of NHDC, the fragment consists of the two aromatic rings of the structure, as the molecule breaks from the two hydroxyl substituted six atom rings through the carbon oxygen bond. These fragments have been observed and reported in the literature when MS/MS is employed [7,8,17-19].

Under these conditions, low instrumental LODs (0.02 – 10 μg/L) and LOQs (0.05 – 15 μg/L) were achieved. The limits obtained are in the same order of magnitude as those in other studies where MS/MS with QqQ was employed [2,5]. The highest limits were obtained for SAC but only because the selected fragment ion did not present a high response compared to the molecular ion.

3.2 Optimization of the SPE

For the SPE procedure, conditioning and loading at pH 3 with HCl was adapted from a previous study in which satisfactory results were reported when using Oasis HLB cartridges [15]. However, elution was optimized for the organic extract to be compatible with the HILIC mobile phase. Thus, the idea was to eliminate the evaporation step and take advantage of the compatibility of HILIC with the organic extract. For this reason, the suitability of the injecting solvent was tested, as peak distortion or changes in retention time may be observed. It was observed that a percentage of MeOH higher than 25% in the sample solvent caused peak distortion in the HILIC separation. Thus, the use of MeOH solutions required subsequent evaporation to redissolve the sample in ACN. Therefore, 25 mL of a solution with the analytes were loaded into the cartridge and then eluted with 5 mL of different solutions: 1) ACN, 2) 10% NH_4OH/ACN, 3) 1:9 MeOH:ACN and 4) 1:5:16 NH_4OH:MeOH:ACN. Other solutions for which evaporation would be needed were also tested for comparison purposes: 5) MeOH, 6) 5% NH_4OH/MeOH, 7) 10% NH_4OH/MeOH.

The use of pure MeOH or ACN or a mixture of the two was not sufficient to elute all of the analytes. The use of NH_4OH was needed to achieve higher recoveries for NEO and STE. This can be explained by the formation of hydrogen bonds between the sorbent and the hydroxyl groups of these two compounds, which is only possible when the –OH is in its protonated form, as sorbent polar functionalities do not have acidic hydrogens, which are not later present at basic pH. Thus, NH_4OH/MeOH and NH_4OH /ACN mixtures were the best option. Of the mixtures mentioned above, ACN solution does not need evaporation and, for this reason, it was selected for eluting the analytes from the cartridge. The only care that must be taken when using this elution solvent is that ASP is not stable in the solution more than 1 hour, so the extract must be injected immediately. Alternatively, dry cartridges loaded with sample can be stored at -4°C when preservation in the middle of the process is needed. In addition, the solubility of GLY in organic solvents is limited so it is preferable to include a portion of water when injected into the LC-HRMS system. However, solutions with this proportion of water

distorted the peak shape of the rest of the compounds. As an alternative, a portion of the extract can be evaporated and redissolved in a mixture of H_2O:MeOH (1:4) with the same volume. However, considering that using this alternative involves injecting the redissolved solution for the sole purpose of quantifying this analyte (since the peaks of some of the other compounds would be distorted), it was decided to eliminate this compound from the target list of the present study. In any case, the chromatographic separation proposed herein is suitable for separating GLY. If GLY determination is desired, the present procedure can be used by changing the injection solvent, as explained above.

Several volumes of eluting solvent (2 to 10 mL) were tested in order to optimize this parameter. It was found that 5 mL of NH_4OH:MeOH:ACN (1:5:16) was optimal for completely eluting the analytes, matching the volume randomly used for the optimization of other SPE parameters. For optimization of the washing step, the solutions tested were 1) 10% MeOH in H_2O, 2) H_2O adjusted to pH 3 and 3) H_2O without adjusting the pH. The best option was the ultrapure water adjusted to pH 3 because losses of analytes were not observed and the matrix effect was lower, probably due to the elimination of salts and highly polar compounds from the matrix [20].

The sample volume was optimized by passing 50, 100 and 250 mL through the cartridges. Up to 250 mL of H_2O could be passed through the cartridges, obtaining recoveries greater than 77% for all of the compounds, except for ASP for which 61% is recovered. However, recoveries of ACE, CYC and ASP for this volume (77% for both ACE and CYC, and 61% for ASP) were slightly lower compared to 100 mL (100% and 97% for ACE and CYC, respectively, and 76% for ASP), due to losses through percolation, because these analytes are quite polar. For this reason, higher volumes were not tested further. When extracting 100 mL of the samples, recoveries for river samples and secondary effluent waste waters were satisfactory, unlike primary influent waters, for which 50 mL were selected to reduce the high matrix effect found. Extraction recoveries ($\%R_{SPE}$) were higher than 76% for secondary effluent water and higher than 74% for all of the compounds in primary influent

water (Table 2). $\%R_{apparent}$ for primary influent waters ranged from 51% (SAC) to 80% (SUC), while values ranged from 57% (ACE) to 74% (SUC) and from 69% to 92% for secondary effluent and river water samples, respectively. Apparent recoveries ($\%R_{apparent}$) shown in Table 2 were calculated from the interpolation of the signal of a sample spiked at the beginning of the analytical procedure in a calibration curve prepared in NH_4OH:MeOH:ACN (1:4:15).

Table 2. Apparent and SPE recoveries, matrix effect and %RSD (n=5) for wastewater samples.

	Effluent (100 mL), spiked at 12.5 μg/L				Influent (50 mL), spiked at 25.0 μg/L			
Compound	$R_{Apparent}$ (%)	R_{SPE} (%)	ME (%)	RSD (%)	$R_{Apparent}$ (%)	R_{SPE} (%)	ME (%)	RSD (%)
ACE	57	95	-40	9	54	74	-27	5
CYC	62	82	-24	25	75	90	-17	13
SAC	74	94	-21	11	51	88	-42	10
ASP	60	76	-21	13	101	99	2	8
SUC	63	105	-40	6	80	105	-24	3
NHDC	62	81	-23	21	74	81	-9	17
STE	59	88	-33	8	60	87	-31	11

The matrix effect (%ME) shown in Table 2 was calculated from the interpolation of the signal of an extracted sample spiked just before injection in the calibration curve mentioned. The concentration obtained (C_{exp}) from this calibration curve was replaced in the following formula $\%ME = (C_{exp}/C_{theo} \times 100\%) - 100\%$. When calculated this way, ME is expressed as the percentage of response that increases or decreases. It must be pointed out that unspiked samples were always considered to subtract blank signals.

In the case of primary influent waters, the ME ranged from -42% to 2% (Table 2), while for secondary effluent and river waters, it ranged from -40% to -21% and from -43% to 5%, respectively. Values indicated the presence of ion suppression due to the complexity of the matrices studied. Enhancement of the

signal was scarcely observed in contrast to the results of the HILIC method reported by Kokotou et al. [5], in which all analytes displayed response enhancement, except for neotame (not included in the present study), SAC and CYC [5].

Even when ion suppression was observed, the ME was lower than in other studies. For instance, in a reversed-phase liquid chromatography (RPLC) approach, Scheurer et al. [1] reported ME values between -77% and 56% for effluent waters when 50 mL was percolated and later reconstituted with 0.5 mL of a 20 mM CH_3COONH_4 in 4:1 H_2O:ACN solution. ME values reported by Ordóñez et al. [2] ranged from -93% to -31% when 50 mL of influent water was preconcentrated to 2 mL and then injected into a HILIC system. The RPLC approach of the same study gave ME values similar on average to the percentages obtained in the present method (Table 2).

3.3 Method validation

Validation of the method proposed was performed by evaluating the linear range, LOQ, LOD and precision (intra-day and inter-day) of the results obtained.

Precision of the concentration values were expressed as relative standard deviation (%RSD, n=5). Extractions were assessed for two levels of concentration within the same day (intra-day) and on consecutive days (inter-day) for the river samples and at one concentration for the primary influent and secondary effluent samples, due to the ubiquity of some of the compounds in these matrices at relatively high concentrations. Repeatability (Table 2) expressed as %RSD (n=5) evaluated on the same day and on consecutive days was < 25% in all cases.

In order to correct the ME, matrix-matched calibration was used for all of the compounds except for ACE, which was present at relatively high concentrations in all of the samples analysed. Due to ACE ubiquity in the samples, quantification at low levels with the matrix-matched calibration was not possible, so an external calibration was considered. For the matrix-matched calibration curves the optimal volume for each sample (influent, effluent and river water) was spiked with different concentrations, extracted and subsequently injected. Five points were considered with spiked concentrations ranging between

Table 3. Detection limits and quantification limits for each type of sample.

	River		Effluent		Influent	
Compound	LODs (µg/L)	LOQs (µg/L)	LODs (µg/L)	LOQs (µg/L)	LODs (µg/L)	LOQs (µg/L)
ACE	0.001[a]	0.003[a]	0.002[a]	0.004[a]	0.004[a]	0.01[a]
CYC	0.001[a]	0.007[a]	0.1	0.25	0.25	0.75
SAC	0.01	0.5	0.7[a]	1[a]	0.25	0.5
ASP	0.25	0.5	0.5	1	0.5	1
SUC	0.1[a]	0.3[a]	0.2[a]	0.4[a]	0.25[a]	0.6[a]
NHDC	0.05	0.1	0.5	1.5	0.1	0.25
STE	0.25	0.5	0.5	0.75	0.5	0.75

[a] Estimated from instrumental limits considering apparent recovery.

LOQs and 100 µg/L. Matrix-matched calibration curves showed satisfactory determination coefficients ($0.9903<R^2<0.9998$) when the linearity of the response was evaluated.

Limits were established as the concentration at which the signal-to-noise ratio was 3 and 10 for the LODs and the LOQs, respectively. When noise was not observable, a signal of magnitude $\sim 5x10^3$ was selected as the LOD, and LOQs were selected as the lowest point of the calibration curve. Detection of the selected confirmatory fragment was always taken into account. Due to the presence of some of the analytes in the blanks, determination of LODs and LOQs of these compounds was difficult. In these cases (ACE, CYC, SAC and SUC), LODs and LOQs were estimated from the instrumental limits taking into account the $\%R_{apparent}$, and results are shown in Table 3.

For primary influent and secondary effluent waters, LODs ranged between 0.002 and 0.7 µg/L, with LOQs between 0.004 and 1.5 µg/L. Lower values were obtained for river water samples, with LOQs not exceeding 0.5 µg/L. These values are of the same order of magnitude as those reported in other studies [14,19,21]. Another study could not be compared with the values presented above because validation was performed for tap water only [1].

3.4 Application to environmental samples

Five samples of both primary influent and secondary effluent STP waters, three samples of both influent and effluent waters of the tertiary process, and five samples of river waters were analysed in duplicate. Detailed information of the concentrations obtained can be found in the Table 4. ACE and SUC were quantified in all of the influent waters in both the primary and tertiary samples. CYC and SAC were found in half of the primary influent samples from the Reus and Tarragona treatment plants. Table 4 also shows the mass error for confirmation purposes and as it can be seen it was lower than 5 ppm in all cases. Levels reported in this paper are similar to those reported in the literature. Kokotou and Thomaidis [5] reported concentrations ranges from 12 to 25 μg/L for ACE, 15 to 25 μg/L for SUC, 6 to 58 μg/L for CYC and 15 to 45 μg/L for SAC in influent waters from STPs in Athens. Ordóñez et al. [2] reported levels ranging from 25.0 to 26.7 μg/L for ACE, 3.0 to 5.3 μg/L for SUC, 25.9 to 36.4 μg/L for CYC and 18.4 to 22.3 μg/L for SAC in waste waters from NW Spain. Scheurer et al. [1] reported concentrations ranges from 34 to 50 μg/L for ACE and SAC, up to 190 μg/L for CYC and below 1 μg

Table 4. Ranges of concentration in μg/L of the samples determined.

Concentration (μg/L)	ACE	CYC	SAC	ASP	SUC
Reus					
influent (n=2)	33.4 - 38.8	68.4 - 108.9	14.4 - 18.2	n.d - n.q	7.2 - 9.1
effluent (n=2)	12.3 - 16.6	n.q - 1.4	n.q	n.d	4.6 - 8.5
Tarragona					
influent (n=3)	23.8 - 31.7	n.q - 58.5	n.q - 19.5	n.d	3.9 - 7.7
effluent (n=3)	5.4 - 7.8	n.d - n.q	n.q	n.d	2.7 - 6.7
Vila-seca					
secondary effluent (n=3)	1.1 - 6.7	n.q	n.d - n.q	n.d	2.7 - 4.3
tertiary effluent (n=3)	n.q	n.q	n.q	n.d	n.d
Mass error (ppm)[a]	2.6	1.1	0.6	-	3.2

n.d. Not detected.
n.q. Detected but below LOQ.
[a]Average obtained for masses measured in the samples.

/L for SUC in influent wastewaters from Germany. Other similar results can be found in several studies [19,21,22]. The results of the present study were slightly different from those obtained in a previous work, in which the levels reported for ACE, SUC and SAC were higher and CYC concentrations were lower [15]. This can be attributed to differences in the sample collection period or differences in the consumption of the sweeteners. A chromatogram of an influent water sample is shown in Figure 2 where the concentration and mass error for each compound is also detailed. In secondary effluents, concentrations ranged from 5.4 to 16.6 µg/L for ACE and 2.7 to 6.7 µg/L for SUC. CYC and SAC were not found in secondary effluent waters, which might be attributed to the removal of these two compounds during the treatment process. This fact has been reported in several studies [1,5,22]. In the case of ACE and SUC, they were poorly eliminated in the treatment process. The results obtained in this study regarding elimination of ACE and SUC are consistent with literature [1,13,23,24].

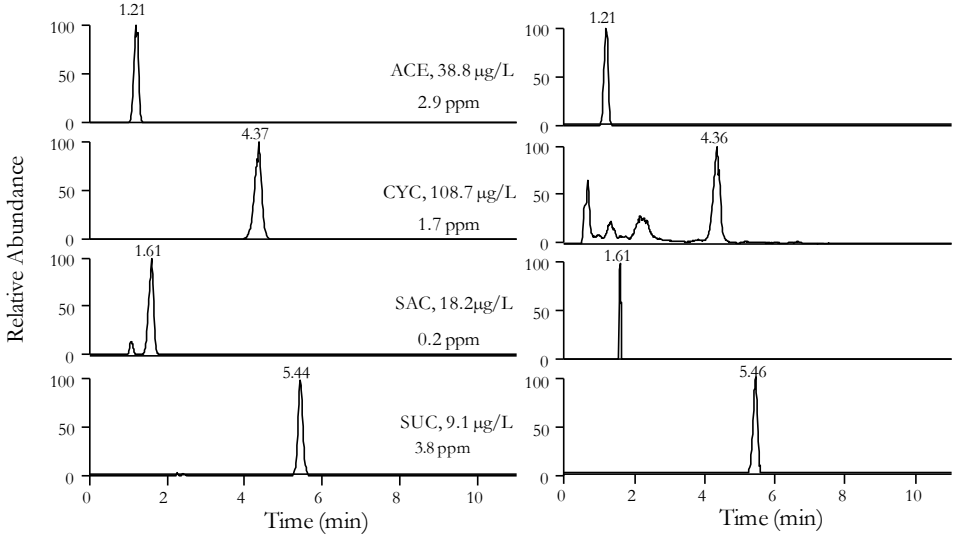


Figure 2. Chromatogram obtained when a blank influent water sample (Influent Reus) was analyzed by SPE HILIC-HRMS. Left: molecular ions. Right: fragments.

With regard to river waters, in all of the samples analysed, ACE, CYC, SAC and SUC were detected below LOQs. ASP, NHDC and STE were not detected in any sample. Only one river sample from the Llobregat River displayed quantifiable levels of ACE, CYC and SUC between 0.03 and 0.57 μg/L. The presence of ACE and CYC in the river may be explained by an accumulation of the low quantities coming from STPs or direct industrial disposal. These levels are similar to those found in some studies. For instance, Gan et al. [17] found concentrations of ACE between 2.7 and 4.7 μg/L and between 0.02 and 0.7 μg/L for the rest of the compounds. Other studies have reported values ranging from 0.05 to 5.8 μg/L [1,2]. In a European screening of SUC including 120 river samples in 23 countries, the concentration of this sweetener was confirmed with concentrations up to around 1 μg/L [11].

4 Conclusions

A method was successfully developed and validated for the determination of a group of artificial sweeteners employing HILIC coupled to HRMS in river and waste water samples. Stevioside, which has never been determined in multi-analyte methods, was included in the method. The zwitterionic stationary phase gave better retention for ACE and SAC compared to the bare silica material under optimized conditions.

Apparent recoveries (overall method) achieved were higher than 51% using Oasis HLB cartridges with a satisfactory matrix effect. Fast sample treatment was possible, as cartridges were eluted with a mixture of NH_4OH:MeOH:ACN (1:4:15) and directly injected into the HILIC-HRMS system achieving great detection and quantification limits.

Four analytes (ACE, CYC, SAC and SUC) were found in different samples from the Tarragona area, mostly present in influent samples.

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

3.1.3. Discussion of results

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Daniela Salas Acosta

The previous studies focused on two different approaches for the study of HILIC as alternative to separate polar compounds, including an investigation to deepen into the retention mechanisms involved and the development of a method applying HILIC coupled to mass spectrometry to analyze complex matrices. The HILIC-HRMS method developed is one of the few studies coupling these two techniques in the environmental field and the first one focused on artificial sweeteners.

One of the most important observations concerning the retention behavior in HILIC was the clear influence of the stationary and mobile phases on the chromatographic retention, separation and selectivity, for which their optimization was very important. The two types of stationary phases tested, this is, bare silica and zwitterionic phases, showed important differences concerning selectivity and retention behavior when changing the mobile phase conditions. As previously discussed, the separation of the iodinated X-ray media selected (ICM) on the bare silica phase was highly affected by changes in pH, type of additives and salt concentration of the mobile phase in contrast with the zwitterionic phase, which was less affected by this changes. The observations made during the study of the predominant retention mechanisms suggested that adsorption played an important role in the retention, so differences between columns could be directly linked to differences in their structures. Both phases may interact with the ICM through Van der Waals forces and hydrogen bonding but the bare silica phase had more binding sites for the formation of hydrogen bonds with the ICM rich in hydroxyl groups, which can be more affected by mobile phase changes. Actually, it was observed that ICM with higher number of hydroxyl groups were more retained, with the exception of diatrizoic acid negatively charged. Experimental observations showed how ICM were more retained at pH 5 and at high salt concentrations. This can be explained because at higher pH values more silanol groups are deprotonated which could lead to a decrease of hydrogen bonds with the analytes (Figure 1). Moreover, an increase in salt concentration can favor the direct interactions of the analytes with the stationary phase by salting-out effect.

In contrast to ICM, the retention of artificial sweeteners was affected by changes in the mobile phase conditions when using both columns. Possibly, direct interactions of the analytes with the stationary phase were less important for the overall retention being hydrophilic partition predominant in this case. Thus, the higher retention observed when working with higher salt concentration could be explained as a consequence of the increase in thickness of the water layer. Furthermore, because some artificial sweeteners are negatively charged under separation conditions, the contribution of ionic interactions is quite feasible for these analytes. Their enhanced retention observed when increasing salt concentration can therefore be attributed to the suppression of ionic interactions at high salt concentration, observing less repulsion from the negative charges present in both stationary phases [1].

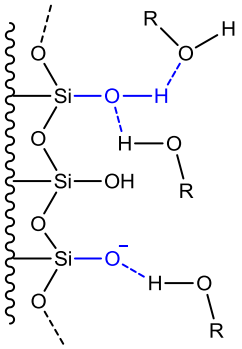

Figure 1. Schematization of active sites susceptible to hydrogen bonding in bare silica phases, being R-OH a compound with hydroxyl groups.

Indistinctly of the type of mechanism driving retention, the most suitable mobile phase conditions selected as optimum in both studies were the same. For the two groups of target compounds, $HCOONH_4$/HCOOH buffered mobile phases at high salt concentrations adjusted at the pH with the highest buffering capacity gave the best results concerning peak shape, retention, selectivity and even reproducibility. In general, the use of $HCOONH_4$/HCOOH additives was better than all tested buffers based on

CH_3COONH_4/CH_3COOH, probably due to differences in elution strength [2].

Whereas the silica stationary phase was the best option for separating the ICM, the zwitterionic phase was preferred for the artificial sweeteners. In both cases though, the zwitterionic phase exhibited higher retention for the target analytes, which was favorable for the separation of the artificial sweeteners poorly retained on the silica phase but detrimental for the separation of the ICM, obtaining analysis too long in this case. In addition, the elution order in the two different phases was very similar for both groups of target compounds. All of these observations could be more accurately explained if further mechanistic studies are performed considering that both ICM and artificial sweeteners have very interesting properties and structures. Certainly, the results found in this Thesis contribute to the general knowledge of this technique, especially because the selected analytes are poorly studied by this LC mode and mechanistic studies are commonly performed on model compounds.

The chromatographic separations achieved in this section clearly show advantages over methods based on reversed-phase LC. For the ICM, separations using C_{18} or RP polar-embedded stationary phases require low flow rates and high water mobile phase proportions in order to promote retention and achieve a suitable separation with satisfactory retention times and analysis time [3]. Under these conditions, peak shape is usually affected as well as ionization in MS-based detectors. For instance, Ens *et al.* [4] separated a group of ICMs and artificial sweeteners in a C_{18} column requiring a high water proportion in the mobile phase and showing little retention, as higher retention time was 7.2 min. In this study, considerable matrix effect was reported even when the matrices had relatively low complexity (surface and drinking water) in comparison to wastewater samples. Recently, Valls-Cantenys *et al.* [5] developed a multi-residue method for the determination of 35 micropollutants in environmental waters, including 5 ICMs also studied in this Thesis. Using a C_{18} phase, these analytes were the compounds firstly eluted even when a flow rate of 0.2 mL/min was used. The separations

presented herein showed great peak shape and satisfactory retention using flow rates at 0.5 mL/min and low water proportion.

Great separation within a short analysis time was obtained for the artificial sweeteners, however, acesulfame and saccharine eluted early. Recently, Kubica *et al.* [6] achieved a HILIC separation where the retention of these two compounds was considerably increased, obtaining retention times higher than 6 min, whereas the ones achieved in our study are 1.23 and 1.56 min, respectively. This enhanced retention was achieved using the Acclaim Trinity P2 column which is based on silica particles whose inner pores are covalently bonded with hydrophilic groups and the surface is coated with nanopolymer particles with anion-exchange properties. Thus, the stationary phase is capable of promoting cation-exchange (through deprotonated silanol groups), anion-exchange, reversed-phase and hydrophilic interactions. The possibility of interacting with the stationary phase trough several mechanisms was responsible for the improved retention of the two sweeteners. It becomes evident how advances on stationary phase development technologies will further improve the capabilities of HILIC for the determination of polar compounds.

Apart from advantages in chromatographic separation, the use of HILIC also helped to shorten the sample preparation procedure and improved MS detection. In the method developed for the artificial sweeteners, one of the greatest advantages was the possibility of directly inject the organic extract obtained from the SPE into the LC-HRMS system. The elimination of the evaporation step highly simplified the sample treatment procedure. As previously discussed, the matrix effect obtained for wastewaters was better compared to other studies as it was around -20% for most of the analytes.

One limitation found during the application of HILIC was the long times required for the equilibration of the stationary phase in order to obtain reproducible retention times. Fortunately, conditioning and equilibration times were optimized and automatically settled. However, strategies to improve this limitation or the use of stationary phases addressing this issue should be explored in future studies.

The development of HILIC separations for both groups of compounds contributed to widen the applications of this LC mode, showing good results and advantages over RPLC. So far, only our group has reported HILIC methods for the separation of ICM while in the case of the artificial sweeteners the number of studies is quite reduced.

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UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

3.2. Alternative applications of mixed-mode ion-exchange polymeric sorbents for polar compounds

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In the previous section, HILIC was presented as an alternative LC mode to retain and separate hydrophilic and polar compounds with environmental interest. However, the problems found for this class of compounds are not only limited to the chromatographic separation but they might also be present during the sample preparation step. As stated in the Introduction, solid-phase extraction (SPE) is the preferred sample treatment technique for liquid samples and a great number of sorbents have been developed to address particular problems found in the existing ones [1]. Polymeric sorbents were developed to solve instability problems of silica-based sorbents at extreme pH values and, after that, hydrophilic character was introduced to these polymeric materials to address lack of retention of polar compounds [2]. However, even hydrophilic polymeric sorbents show limited retention for very polar or ionic compounds, which motivated the introduction of mixed-mode ion-exchange sorbents. As they are functionalized polymers with ionic groups, they can promote reversed-phase and ionic-exchange interactions together in the same cartridge [3, 4]. Thus, this class of sorbents represents the most recent advance focused on improving retention of very polar compounds combining better capacity and selectivity.

Depending on the protocol selected for these sorbents, they can be "universal" because they can retain together neutral, basic and acidic compounds with different polarities, or they can be very selective toward ionic compounds, eliminating neutral analytes and compounds with the same charge as the sorbent, in washing steps using pure organic solvents. The first approach has lately been mostly used in omic applications to ensure the preconcentration of the highest number of analytes caring not to rule out ionic or very polar compounds of interest [5]. For specific target compounds when using MS-based detectors, the second type of SPE procedure is more appealing, as the elimination of interferences should reduce the matrix effect often observed in ESI sources [6].

Several environmental, biological or food applications of mixed-mode ion-exchange sorbents have reported their advantages in comparison to others, like enhanced retention for particular ionic or very polar analytes, selectivity

and clearer extracts with reduced matrix effect [3, 4, 7]. Nevertheless, many of these studies make use of the protocol recommended by the manufactures without further optimization or study of the effect of the different SPE conditions. The understanding of the interactions between the analytes and the sorbents, as well of the influence of parameters such as pH, volume or type of solvent during each SPE step is very important to take profit of the potential of these materials. In addition, there are several compounds which could benefit from the use of mixed-mode sorbents that has not been evaluated yet. For these reasons, the evaluation and application of these materials is an active research topic.

In the first study included in this section, commercial mixed-mode sorbents were applied to a group of contaminants used in the industry, benzotriazole, benzothiazole and benzenesulfonamide derivates, which bare interesting structural properties as the capability of delocalizing electron density. The relevance of this study relied on the development of a successful and selective SPE protocol using these materials for a group of analytes that were actually in their neutral state during the extraction. The ionic retention of these compounds was compared in mixed-mode sorbents with strong cation and anion-exchange properties using a procedure with a methanol washing step to eliminate interferences. After the study of the influence of some SPE parameters and the comparison of both types of sorbents in ultrapure water, two methods with each material were developed and further validated in environmental waters using LC-HRMS for the analysis.

As discussed in the Introduction, these sorbents can be highly selective if a washing step with pure organic solvent is included to remove interfering compounds, while the ionic analytes are kept retained through ion-exchange interactions. This methodology is limited to groups of ionizable compounds with the same charge though, because sorbents are functionalized with either positively or negatively charged groups being capable of ionically interacting with only one type of ions. However, several applications require the simultaneous extraction of basic and acidic compounds from complex samples. One alternative for simultaneous extraction purposes is the collection of the methanol washing fraction in addition to the elution

fraction, to obtain cations and anions in separated solutions with lower complexity, which can be analyzed in two different runs expecting to have less interfering compounds in each injection. The problem with this approach is that the analysis time duplicates and neutral interferences are not eliminated. Some studies have placed mixed-mode sorbents with cation and anion-exchange properties in series but proposed protocols were long or also involved the collection of the analytes in different fractions [8, 9].

The second study included here combined for the first time mixed-mode sorbents in pairs of opposite charge in only single cartridge. When using cartridges in tandem, two independent protocols must be performed while a single protocol is required when combining charges in the same cartridge. In particular, four individual sorbents with different properties (strong cation-exchange, SCX, strong-anion exchange, SAX, weak cation-exchange, WCX and weak anion-exchange, WAX) were combined in different configurations to obtain four cartridges containing positive and negative charges together. A list of basic, neutral and acidic pharmaceuticals was selected as model compounds to evaluate the performance of the four cartridges. Information regarding their behavior on individual mixed-mode ion-exchangers had already been gathered from previous results. The influence of several SPE parameters was studied on the interactions analyte-sorbent as well as the strong or weak properties of the materials and their proportions (equimolar or same weight). The optimized SPE procedure was further evaluated on environmental waters to study the performance in matrices of high complexity.

The results obtained in both of these studies have been published in the *Journal of Chromatography A* 1444 (2016) 21-31 or submitted for publication in the same journal.

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3.2.1. Study of the retention of benzotriazoles, benzothiazoles and benzenesulfonamides in mixed-mode solid-phase extraction in environmental samples

UNIVERSITAT ROVIRA I VIRGILI
STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

STUDY OF THE RETENTION OF BENZOTRIAZOLES, BENZOTHIAZOLES AND BENZENESULFONAMIDES IN MIXED-MODE SOLID-PHASE EXTRACTION IN ENVIRONMENTAL SAMPLES

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Abstract

In the present study, the capabilities of strong cation-exchange and strong anion-exchange sorbents for solid-phase extraction (SPE) have been evaluated for the selective retention of benzotriazoles (BTRs), benzothiazoles (BTs) and benzenesulfonamides (BSAs), which are a group of neutral analytes with interesting properties such as high polarity and the capability of delocalizing electron density. The retention of these analytes has been compared in both sorbents for the first time, using a SPE procedure specially designed to promote ionic retention of the analytes with the objective of including a washing step with an organic solvent to eliminate interferences retained by hydrophobic interactions.
As a result, ionic interactions between the analytes and both sorbents were observed, which allowed the successful introduction of a washing step using methanol in the SPE procedure even when most of the analytes were in their neutral state under SPE conditions. Consequently, a method was developed and further validated for each sorbent using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). Apart from the development of an improved method, special attention was paid to the discussion of the interactions present between the sorbents and each group of analytes to explain how these analytes in their neutral state might develop ionic interactions with the sorbents. At the end, the use of these sorbents helped to simplify previous developed methods where hydrophobic/hydrophilic sorbents were used, obtaining enhanced results when evaluated in river water and effluent and influent wastewaters.

Keywords: *benzotriazoles; benzothiazoles; benzenesulfonamides; mixed-mode solid-phase extraction; high-resolution mass spectrometry; environmental waters.*

1 Introduction

Mixed-mode polymeric sorbents have been developed to be applied in solid-phase extraction (SPE) to address the limitations of hydrophobic/hydrophilic sorbents. These sorbents have a polymeric backbone capable of retaining compounds through reversed-phase interactions and ionic groups capable of retaining ions with the opposite charge so both types of interactions are combined in one sorbent. The ionic groups bonded to the polymer are commonly sulfonic or carboxylic acids groups, which promote strong cation-exchange (SCX) and weak cation-exchange interactions (WCX), respectively; or quaternary amine groups for strong anion-exchange interactions (SAX); or tertiary or secondary amine groups for weak anion-exchange interactions (WAX) [1, 2].

The presence of these ionic interactions allows basic or acidic compounds to be selectively extracted from complex matrices. These sorbents have been applied to extract several groups of compounds such as pharmaceuticals, drugs of abuse, herbicides, and biological compounds, among other analytes, from several biological, foodstuff and environmental matrices [1, 3-9]. Generally, the methods are developed to extract the ionic analytes selectively in the elution step, which can be eluted separately from interferences that are rinsed in previous washing steps. To do so, the selection of pH values and solvents is very important and it depends on the type of sorbent and the structure of the analyte.

Benzotriazoles (BTRs), benzothiazoles (BTs) and benzenesulfonamides (BSAs) include substances containing the skeleton of benzotriazole (BTR), benzothiazole (BT) and benzenesulfonamide (BSA), respectively (Table 1), and they are considered emerging contaminants with particular chemical structures [10]. Both BTRs and BTs are heterocyclic compounds comprising two fused aromatic rings, allowing the delocalization of the electron density throughout the entire molecule. Moreover, the presence of electronegative atoms in one of the rings introduces high polarity to the structure. Furthermore, BTR can exist as two tautomers and can give resonance-stabilized anions and cations when treated with strong bases and electrophiles, such as all azoles and their bicycle derivatives [11]. In the case of BSAs,

delocalization of the electrons only occurs on the benzene ring but they are very polar compounds thanks to the sulfonamide group. The properties of these three groups of compounds have become important in several industrial applications and, for this reason, they have become high production volume substances. Their applications include: flame and corrosion inhibitors, photosensitizers, intermediates in the production of pharmaceuticals and dyes, de-icing/anti-icing fluids, herbicides, fungicides and chemotherapeutic applications [12-15]. However, these compounds can reach environmental waters and, for this reason, they have been classified as emerging organic contaminants [10, 16, 17]. A number of toxicity studies have shown that BTR, 4-methyl-1-H-benzotriazole (4TTR) and 5-methyl-1-H-benzotriazole (5TTR) (these two usually measured as a total and referred to as tolyltriazole, TTR) are hazardous to plants. BTR is also mutagenic in some bacteria cell systems, potentially estrogenic in fish and a suspected human carcinogen, while TTR has been reported to be toxic to microorganisms [18].

The technique of choice for determining these compounds in environmental waters is liquid chromatography (LC) coupled to mass spectrometry in tandem (MS/MS) or high resolution mass spectrometry (HRMS) [10, 12-14, 19]. With regard to the sample treatment, most of the methods developed to determine BTRs, BTs and BSAs in these matrices use SPE with polymeric hydrophobic/hydrophilic sorbents (such as Oasis HLB or Strata-X) for the extraction of the analytes [10, 12-14, 20-23]. However, the retention that is achieved for the target analytes when using this type of sorbents is also gained by the interferences present in the matrix and, as a result, the methods proposed to determine these compounds exhibit a considerable matrix effect (ME) [10, 12, 13, 15, 19, 23]. For methods where ME was successfully reduced [20], the SPE procedure demanded an additional washing step that extended sample treatment and increased costs. For this reason, some attempts have been made to reduce this ME by using anion-exchange mixed-mode sorbents that claim to present more selectivity for anions [19, 20]. In one case [19], the analytes were eluted with a mixture of organic solvents leaving the acidic interferences retained, a procedure that showed good results for river water and effluent wastewater but was less satisfactory for influent

wastewater. In another study [20], the sorbent was dismissed because results of preliminary tests were not promising.

In the present study, the strong anion-exchange mixed-mode sorbent Oasis MAX is evaluated focusing its capabilities on selectively retaining a group of BTRs, BTs and BSAs, using conditions to promote ionic interactions between the sorbents and the analytes. Also, for the first time, the strong cation-exchange mixed-mode sorbent Oasis MCX is evaluated for these analytes and compared with the Oasis MAX, because ionic interactions with the analytes are feasible thanks to the structural properties of the analytes mentioned above. The aim is to reduce the ME compared with other methods but also to increase the knowledge and understanding for these types of sorbents as the selected analytes bare interesting properties. The two methods using Oasis MAX or Oasis MCX in SPE followed by LC-HRMS are developed and further validated.

2 Experimental

2.1 Reagents and standards

Solid standards of 1-H-benzotriazole (BTR) and four of its derivatives: 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); together with benzothiazole (BT) and its derivatives: 2-aminobenzothiazole (NH_2BT), 2-hydroxybenzothiazole (OHBT) and 2-(methylthio) benzothiazole (MeSBT); and benzenesulfonamide (BSA) and the four derivatives: 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, Missouri, USA). Stock solutions of 1 mg/mL were prepared by dissolving each solid standard in methanol (MeOH) and were stored at -20°C. All working solutions of a mixture of all compounds were prepared weekly in ultrapure water and were stored at 4°C in the dark. The structure of the analytes, pK_a values, log P values and masses of the molecular ions and the selected fragments are shown in Table 1.

Ultrapure water was obtained using a water purification system (Veolia, Sant Cugat del Vallès, Spain) and ultra-gradient HPLC grade MeOH and acetonitrile (ACN) were purchased from J.T.

Baker (Deventer, The Netherlands). Acetic acid (CH_3COOH) from SDS (Peypin, France), formic acid (HCOOH) from Sigma-Aldrich, hydrochloric acid (HCl) from Scharlab (Barcelona, Spain) and ammonium hydroxide (NH_4OH) from Panreac (Barcelona, Spain) were used to prepare the mobile phase and the solutions for the SPE.

2.2 Sampling

Influent and effluent wastewater samples were collected from a Tarragona sewage treatment plant which operates with a primary and a secondary treatment. River water samples were collected from the River Ebre in Catalonia. Water samples were collected in pre-cleaned bottles and were then stored at -20ºC. Prior to analysis, the samples were filtered through a 0.45 μm glass-fibre filter (Fisherbrand, Loughborough, UK). The pH of the samples was adjusted to 3 or 7 with HCOOH before the SPE procedure.

2.3 Solid-phase extraction procedure

Oasis MCX and Oasis MAX cartridges (500 mg) supplied by Waters (Milford, MA, USA) were used. The optimal conditions were: loading volume was 250 mL for river water and effluent wastewaters, and 100 mL for influent wastewaters.

Oasis MCX cartridges were conditioned with 10 mL of MeOH, followed by 10 mL of ultrapure water adjusted to pH 3 with HCOOH. Samples were also adjusted to pH 3 before being loaded into the cartridge. Two consecutive washing steps were used: 1) 5 mL of 5% HCOOH in aqueous solution and 2) 2 mL of pure MeOH. Finally, the analytes were eluted with 5 mL of 5% NH_4OH in MeOH.

Oasis MAX cartridges were conditioned with 10 mL of MeOH, followed by 10 mL of ultrapure water adjusted to pH 7. Samples were loaded at pH 7 and the washing steps were as follows: 1) 5 mL of 5% NH_4OH in aqueous solution and 2) 2 mL of pure MeOH. For the elution, 5 mL of 5% HCOOH in MeOH were used. When analyzing water samples, the extracts (in 5% HCOOH or 5% NH_4OH in MeOH solutions) obtained after the elution were evaporated up to ~250 μL and taken to a final volume of 1 mL with ultrapure water.

Table 1. Chemical structure, pK_a, log P, and exact masses of the studied analytes.

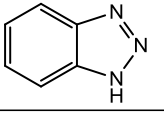
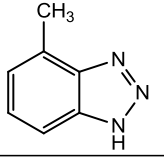
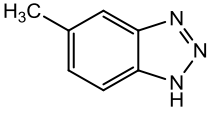
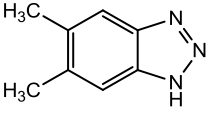
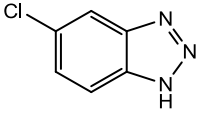
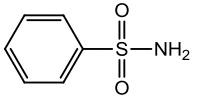
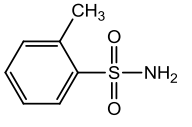
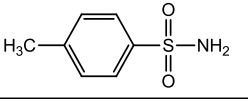
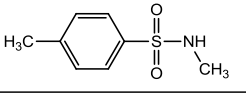
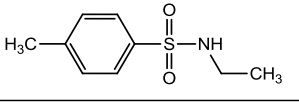
Compound	Formula	Structure	pKa[a, b]	log P[a]	Molecular ion (m/z)	Fragment Formula (exact mass, m/z)
Benzotriazole (BTR)	$C_6H_5N_3$		8.38	1.44	$[M+H]^+$ 120.05562	$C_5H_5^+$ (65.03858) $C_6H_6N^+$ (92.04948)
4-methyl-1H-benzotriazole (4TTR)	$C_7H_7N_3$		8.74	1.82	$[M+H]^+$ 134.07127	$C_6H_7^+$ (79.05423) $C_6H_5N_2^+$ (105.04472) $C_6H_5^+$ (77.03858)
5-methyl-1H-benzotriazole (5TTR)	$C_7H_7N_3$		8.74	1.98	$[M+H]^+$ 134.07127	$C_6H_7^+$ (79.05423) $C_6H_5N_2^+$ (105.04472) $C_6H_5^+$ (77.03858)
5,6-dimethyl-1H-benzotriazole (XTR)	$C_8H_9N_3$		8.92	2.28	$[M+H]^+$ 148.08692	$C_7H_7^+$ (91.05423) $C_7H_9^+$ (93.06988) $C_6H_5N_2^+$ (105.04472)
5-chloro-1H-benzotriazole (ClBTR)	$C_6H_4ClN_3$		7.46	2.13	$[M+H]^+$ 154.01665	$C_5H_4Cl^+$ (98.99960) $C_6H_4N^+$ (90.03382) $C_5H_4{}^{37}Cl^+$ (156.01370)

Table 1. (Cont.).

Compound	Formula	Structure	pKa[a, b]	log P[a]	Molecular ion (m/z)	Fragment Formula (exact mass, m/z)
Benzothiazole (BT)	C_7H_5NS		0.85	1.90	$[M+H]^+$ 136.02155	$C_6H_5S^+$ (109.01065) $C_5H_5^+$ (65.03858)
2-amino benzothiazole (NH_2BT)	$C_7H_6N_2S$		3.94	1.88	$[M+H]^+$ 151.03244	$C_6H_5S^+$ (109.01065) $C_5H_5^+$ (65.03858) $C_6H_6SN^+$ (124.02155)
2-hydroxy benzothiazole (OHBT)	C_7H_5NOS		10.41	1.81	$[M+H]^+$ 152.01646	$C_6H_6SN^+$ (124.02155) $C_6H_6N^+$ (92.04948) $C_6H_5S^+$ (109.01065)
2-(methylthio) benzothiazole (MeSBT)	$C_8H_7NS_2$		1.22	2.84	$[M+H]^+$ 182.00927	$C_7H_5S_2N^+$ (166.98579) $C_6H_5S^+$ (109.01065)

Table 1. (Cont.).

Compound	Formula	Structure	pKa[a, b]	log P[a]	Molecular ion (m/z)	Fragment Formula (exact mass, m/z)
Benzene sulfonamide (BSA)	$C_6H_7NO_2S$		10.08	0.49	$[M-H]^-$ 156.01247	$C_6H_6N^-$ (92.05057) SO_2NH^- (78.97335)
o-toluene sulfonamide and p-toluene sulfonamide (TSA)	$C_7H_9NSO_2$		10.17	1.13	$[M-H]^-$ 170.02812	$C_7H_8N^-$ (106.06622) SO_2NH^- (78.97335)
			10.20	0.97		
n-methyl-p-toluene sulfonamide (Me-*p*-TSA)	$C_9H_{13}NSO_2$		11.86	1.91	$[M+H]^+$ 186.05832	$C_7H_7^+$ (91.05423)
n-ethyl-p-toluene sulfonamide (Et-p-TSA)	$C_8H_{11}NSO_2$		11.67	1.91	$[M+H]^+$ 200.07398	$C_7H_7^+$ (91.05423)

[a] Scifinder Database: Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs)
[b] In all cases the pK_a values indicated are referred to the loss of the proton from the neutral form with the exception of BT, NH_2BT and MeSBT for which the pK_a values are referred to the loss of the proton from the conjugated acid.

2.4 Liquid-chromatography-high resolution mass spectrometry (LC-HRMS)

Chromatographic analysis was performed with an Accela 1250 UHPLC system from Thermo Scientific (Bremen, Germany) that includes an automatic injector (Accela Autosampler) and a quaternary pump capable of reaching up to 1,250 bar. The LC system was coupled with an Exactive Orbitrap™ mass spectrometer (also from Thermo Scientific) with a heated electrospray ionization (HESI) source and a HCD collision cell for the fragmentation of the analytes for their confirmation. The chromatographic column used was the Ascentis Express C_{18} (100 mm x 2.1 mm i.d., 2.7 μm particle size, Fused-Core®) supplied by Sigma-Aldrich. The mobile phase was a mixture of solvent A (0.1% CH_3COOH in H_2O/ACN 98:2, v/v) and solvent B (MeOH). The gradient profile started with 0.5% of B and was held for 5.25 min, before being increased to 18% B in 3.5 min and held again for 1.25 min with a total time of 10 minutes. Subsequently, it was increased to 35% B in 6 min and then increased again to 100% in 4 min and held for 4 min before returning to the initial conditions in 1 min. The flow rate was 800 μL/min, the temperature of the column oven was set at 50°C and the injection volume was 20 μL.

In the HRMS instrument, the signal of the molecular ion, $[M+H]^+$ or $[M-H]^-$, of each analyte was monitored to optimize the interface conditions in order to obtain the highest response for all of the analytes. This optimization was performed in full scan at high resolution (50,000 FWHM) in a mass range of 100 to 1,000 m/z. The optimal parameters for positive ionization were: skimmer voltage, 20 V; capillary voltage, 37.5 V and tube lens voltage, 90 V. In the case of negative ionization, they were: skimmer voltage, -25 V; capillary voltage, -40 V and tube lens voltage, -90 V. Other parameters were the same for both ionization modes: spray voltage, 4 kV; sheath gas, 55 AU (adimensional units); auxiliary gas, 20 AU; heater temperature, 400°C; capillary temperature, 350°C; and probe position adjustment: side to side, 0, vertical D and micrometer, 1.25.

For data acquisition, four time windows were used: two in negative mode (0-2.3 and 4.8-7.6 min) and two in positive mode (2.3-4.8 and 7.6-20 min) alternating two consecutive scan

events each window. In all of the windows, the first scan event was a full scan at 50,000 FWHM with 250 ms of injection time while the second scan event was a fragmentation scan at 10,000 FWHM with 50 ms of injection time, using an optimum collision voltage of 25 eV in the HCD cell in all windows. For quantification, the molecular ions were measured (with a mass extraction window of 5 ppm) and, for confirmation, the selected fragments and the ion ratios were taken into account.

3 Results and discussion

3.1 LC-HRMS conditions

LC-HRMS conditions were adapted from a previous study [20] in which the analytes were determined in sewage sludge. The separation of some of the compounds is very difficult, so the most important parameters that were optimized in this previous study should be emphasized: the chromatographic column was also a Fused-Core® type; the orto and para isomers of the toluenesulfonamide (o-TSA and p-TSA) were determined as a mixture named TSA because chromatographic separation was not possible under optimal LC conditions. The separation of 4TTR and 5TTR was also very complex so, in order to obtain the best separation in this case, a percentage of 2% of ACN was added to the aqueous mobile phase and the oven temperature was increased to 50°C. In addition, the initial segment of the gradient profile was kept at low concentrations of organic solvent. The optimal parameters are detailed in Section 2.4 and the fragments selected for confirmation are described in Table 1. The instrumental limits of detection (ILODs) were the concentrations at which the signal to noise ratio (S/N) was 3 for the response of the fragment that showed the highest signal under collision conditions. The obtained values ranged between 1 and 10 μg/L. Instrumental limits of quantification (ILOQs) were the concentration corresponding to the first point of the calibration curve and they ranged from 1 to 25 μg/L (Table S1). Satisfactory linearity was observed in the range between the ILOQs and 1000 μg/L.

3.2 Mixed-mode solid-phase extraction

BTRs, BTs and BSAs are compounds with relatively high polar character due to the presence of the triazole, thiazole and sulfonamide groups, and also

the first two groups have an important delocalized electron density [11]. With this in mind, two commercially available strong ion-exchange mixed-mode sorbents, Oasis MAX and Oasis MCX, were selected to evaluate their capability of establishing ionic interactions with the analytes induced by the strong character of the quaternary amine or the sulfonic group of the sorbents. Weak ion-exchange sorbents were not selected because their functional groups are not permanently charged (as is the case with the strong ion-exchange sorbents) and pH conditions not only affect the analytes but also the sorbent, adding more parameters to be controlled. Furthermore, preliminary tests showed no selective ionic interactions with any of the analytes.

Because of the chemical differences between the strong anionic and cationic exchange sorbents, the conditions for the SPE procedure also differ from each other [24]. When using anionic sorbents, a neutral pH value must be used to load the sample because the analytes must be in their anionic form to establish ionic interactions with the quaternary amine of the sorbent [7]. Likewise, when using cationic sorbents, the analytes must be loaded in their cationic form (usually adjusted to pH 3) to allow interaction with the sulfonic acid groups [25, 26]. The formation of strong ionic interactions allows the possibility of adding different washing steps to the SPE procedure in order to clean the extracts from interferences. An aqueous wash at the selected pH can be used to eliminate the most water-soluble compounds that are poorly retained in the sorbent by hydrophobic interactions and further activates the ionic interactions between the ionic compounds and the sorbent. Subsequently, the second washing step with an organic solvent is added to rinse all of the neutral compounds that are only retained by hydrophobic interactions. Finally, an elution step is needed to turn the analytes back to their neutral state using organic solutions at suitable pH conditions.

The performance of both sorbents and the differences in the ionic retention of the selected analytes will be discussed in the following sections.

3.2.1 Cation-exchange sorbent

Based on the most commonly used conditions, the following starting procedure was selected to optimize the SPE conditions in this sorbent: loading 100 mL of ultrapure water adjusted to pH 3 spiked at 25 μg/L with the mixture of analytes; two washing steps and elution as described in Section 2.3. It should be highlighted that preliminary studies without the washing step showed good recoveries (between 87% and 115%) for all of the analytes.

The starting conditions used for the Oasis MCX sorbent succeeded in retaining and consequently eluting the compounds which was surprising because all of the analytes were in their neutral state, with the exception of NH_2BT which was positively charged (Table 1). In order to evaluate its effect on retention, the pH of the sample was adjusted to 2 (using HCl) before being loaded into the cartridge, rather than 3 as initially established. As a result, no significant differences were observed between both pH values. These observations agree with the fact that all of the analytes remain unchanged at both pH 2 and 3, according to their pk_a values. In order to avoid using relatively extreme conditions, pH 3 was selected as optimal.

Table 2 shows the recoveries obtained in the washing and elution fractions when using the starting SPE conditions described above. BSAs eluted in the second wash fraction corresponding to the pure MeOH wash. Clearly, these compounds interact with the sorbent through hydrophobic interactions which is expected as they are in their neutral state at pH 3. In contrast, BTRs and BTs were completely retained during the washing step and rinsed in the elution fraction even when most of them were also in their neutral state. These compounds seem to be retained to the sorbent through ionic interactions that are more significant than hydrophobic interactions. It was interesting to observe these differences between groups of analytes with regard to their interactions with the sorbents, because one of the aims of the present study was to explore the capabilities of these sorbents for these compounds with interesting properties. It can be observed that the analytes with the capacity of delocalizing electron density (BTRs and BTs, Figure 1) were able to stabilize partial electron charges to interact with the ionic group of the sorbent, while BSAs behaved as neutral compounds, probably because they have no ability to delocalize electron density. In a

subsequent test, a higher volume of MeOH (5 mL) was employed for the second wash. Table 2 also details the distribution of the analytes between both the washing fraction (when using 2 or 5 mL of MeOH) and the eluting fraction. When increasing the volume of MeOH, ClBTR and OHBT were rinsed in the washing fraction instead of the elution fraction, probably because weaker ionic interactions were involved. The electronegativity of the groups attached to the BTRs and BTs seems to have an important effect on the ionic interactions that are formed. In this case, this effect might be detrimental, because retention was decreased, as shown by ClBTR, with the presence of the chloride group, and by OHBT, with oxygen being more electronegative than nitrogen and

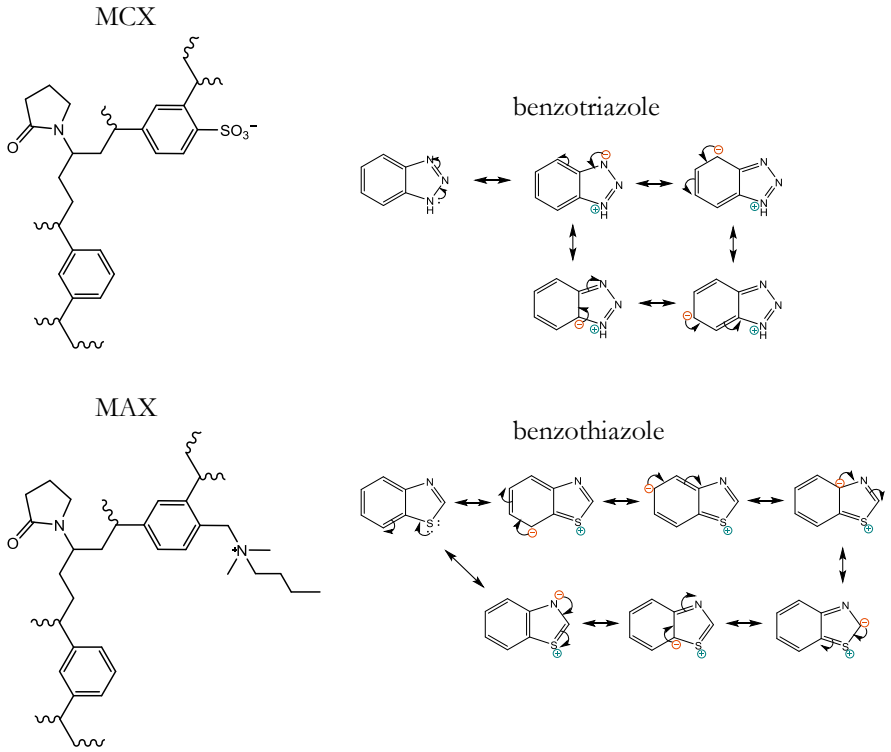


Figure 1. Structure of the Oasis MCX and Oasis MAX sorbents and resonance structures of benzotriazole (BTR) and benzothiazole (BT).

Table 2. % Recoveries or % R_{SPE} obtained when 100 mL ultrapure water were percolated in Oasis MCX and Oasis MAX sorbents using either 2 mL or 5 mL of MeOH in the washing step.

	Oasis MCX				***Oasis MAX***			
	2 mL MeOH		**5 mL MeOH**		**2 mL MeOH**		**5 mL MeOH**	
	w	**e**	**w**	**e**	**w**	**e**	**w**	**e**
BTRs								
BTR		95		99		103		97
4TTR		93		110		101		100
5TTR		97		92		111		106
ClBTR		99	100			91		101
XTR		103		107		107		108
BTs								
BT		73		115		89	90	
NH_2BT		90		85		88	111	
OHBT		60	110			104		104
MeSBT		102		114		104		73
BSAs								
BSA	86		84			88	73	
TSA	80		87			102	80	
Me-p-TSA	78		109		35	77	104	
Et-p-TSA	80		99		47	68	105	

sulfur. Thus, 2 mL of MeOH was selected as second washing step.

As the analytes showed satisfactory recoveries (60% to 115%), a higher loading volume (250 mL) was tested using 2 mL of MeOH in the second washing step. When increasing the loading volume, all analytes showed similar recoveries, however, MeSBT was more evenly distributed between the washing and eluting fraction, so recovery in the elution fraction decreased (65%). For this reason, higher volumes were not further tested and 250 mL was established as the highest loading volume to be tested in environmental waters. Higher volumes of eluting solvent were not tested further because it was observed that the volume initially selected (5 mL) was enough to elute the analytes completing the mass balance of the overall SPE procedure.

3.2.2 Anion-exchange sorbent

In the case of this sorbent, the following starting procedure was selected: loading 100 mL of ultrapure water adjusted to pH 7 spiked at 25 μg/L with the mixture of analytes; two washing steps and elution as described in Section 2.3. Preliminary studies without the washing step showed good recoveries (between 85% and 106%) for all of the analytes.

For this sorbent, the starting conditions were also successful in retaining and consequently eluting the analytes, even when all of them were in their neutral state at the selected pH. As before, the pH of the sample was changed to evaluate its effect on retention. The sample was adjusted to pH 11.5 (with NaOH) instead of 7 as established in the starting conditions, because most of the BTRs, BTs and BSAs (except for BT, NH_2BT and MeSBT) can donate a proton at pH values between 8 and 11, depending on the analyte (see pK_a values in Table 1). Theoretically, retention should be higher at pH 11.5 for those compounds that donate a proton to become negatively charged. However, no differences were observed between the two pH values, so apparently the interactions occurring during the extraction are independent from the donation of the proton. This further confirms that retention is probably controlled by inducing charges in the analytes that are stabilized by resonance rather than actual ion-exchange. Thus, it was decided to adjust the samples to pH 7 in order to avoid using extreme pH conditions.

When using the starting procedure for the Oasis MAX sorbent, all of the analytes were recovered in the eluting fraction, except for Me-p-TSA and Et-p-TSA, which were distributed between both the washing and eluting fractions (Table 2). When the volume of the pure MeOH washing step was increased to 5 mL, all of the BSAs were collected in this washing fraction, together with NH_2BT and BT. Thus, similar to the Oasis MCX sorbent behavior, as BSA has no delocalized charges, they are not able to be retained in any of the mixed-mode sorbents through ionic-exchange mechanisms. Considering the lack of ionic interactions between both sorbents and the BSAs, it was decided to eliminate them from the target list and develop a more selective method for the compounds for which the sorbents display ionic interactions, which is the case of the BTRs and BTs. The latter have the property

of delocalizing the electron densities throughout both aromatic rings that might induce dipoles that ionically interact with the amine groups of the sorbent. Compared to Oasis MCX, the Oasis MAX sorbent showed less retention for NH_2BT and BT (eluting during washing when employing 5 mL of MeOH), while it presented higher retention for ClBTR and OHBT (Table 2). In the Oasis MAX, the electronegativity of the groups attached to the analytes seems not to have a negative effect on the ionic interactions responsible for retention, as ClBTR and OHBT were retained when washing with 5 mL of MeOH. BT showed less retention than its derivatives substituted with electronegative functional groups, suggesting that the effect might actually be favorable. The behaviour of NH_2BT might be explained separately from the rest, because this compound is protonated at pH 3 (pK_a 3.94 in Table 1) but it is neutral at pH 7, explaining the increased retention observed in Oasis MCX, in contrast to the decreased retention observed in Oasis MAX. These results are in agreement with a previous study [20] were Oasis MAX was compared with the hydrophilic/hydrophobic sorbent Oasis HLB. Even when pH and sorbent weight differ from the present study, the loss of BSAs, NH_2BT and BT was also observed in the washing fraction of 6 mL of MeOH.

As BTRs and BTs were not lost using 2 mL of MeOH in the second wash, this volume was used for further optimization, but it was compared again using environmental samples, as will be discussed in the following sections. A higher sample volume (250 mL) was tested and similar results were obtained to those with 100 mL recoveries, except for MeSBT, which was partially rinsed in the washing fraction. For this reason, no higher volumes were tested further.

As can be seen, the ionic interactions promoting the retention of BTRs and BTs on Oasis MCX and Oasis MAX sorbents are not based on ion-exchange per se [27], as these molecules do not develop a clear ionic charge under SPE conditions. This affirmation is evidenced by the fact that different pH values of the loading sample showed no significant differences. However, the analytes are capable of developing partial charge densities that might easily interact with the ionic groups of the sorbents, allowing the

retention of the analytes even when a MeOH washing step was used. This capability of delocalizing electron density throughout the whole molecule is due to the conjugation of both aromatic rings (Figure 1). Some observations suggest that the strong character of the ionic groups present in the sorbents plays an important role in the promotion of the ionic interactions between the analyte and the sorbent. These groups might induce ion-dipole interactions that are feasible thanks to the capabilities of the analytes to stabilize partial charges. As a result, these sorbents showed the capacity of retaining the selected compounds through ionic interactions in spite of being neutral under SPE conditions. When using 2 mL of MeOH as a second washing step, both sorbents showed similar results being slightly better for the Oasis MAX. However, their behaviour with environmental samples might lead to a clearer comparison and for this reason they will be further compared in the following section.

3.2.3 Evaluation in environmental samples

In this section, the previously described SPE procedure was tested on river water and wastewaters in order to evaluate the %ME obtained using HESI in LC-HRMS, with these more selective sorbents. Both sorbents were initially tested in influent wastewater because differences in ME would be more evident in such a complex matrix. The procedure followed was the same as previously described for ultrapure water, but after the elution, the extracts in 5% HCOOH or 5% NH_4OH in MeOH solutions were evaporated up to ~250 μL and taken to a final volume of 1 mL with ultrapure water. Evaporation to dryness was not possible as BT and MeSBT were lost because of their volatility, but it was observed that no losses were found when evaporating up to ~250 μL.

As expected, the extraction was affected by the sample matrix and therefore the volume of sample loaded into the cartridges was reduced from 250 mL to 100 mL. Table 3 shows the results obtained for both sorbents when percolating 100 mL of influent wastewater spiked at 5000 ng/L with the mixture of BTRs and BTs and adjusted to pH 3 (Oasis MCX) and to pH 7 (Oasis MAX).

The recovery named as $\%R_{SPE}$ expresses the yield of the extraction procedure only and it

was calculated as the ratio between the concentrations obtained for samples spiked before and after the SPE procedure, respectively, both calculated from a calibration curve prepared with pure standards. The ME was calculated as $\%ME = [(C_{exp}/C_{theo})*100] - 100$, where the C_{exp} was the concentration of analytes when the sample was spiked after the SPE procedure (just before injection into the LC-HRMS system) calculated from the interpolation in a calibration curve prepared in pure standards; and the C_{theo} was the theoretical concentration of analytes in the final volume (1 mL). The value obtained refers to the percentage of the response that is suppressed or enhanced. The recovery, referred to as $\%R_{apparent}$, was calculated by interpolating the response of the analytes when the sample was spiked before the SPE in a calibration curve prepared in pure standards. This recovery indicates the losses of the overall analytical procedure including the extraction itself and the influence of the matrix in the response. In every case, response of the analytes present in the blanks was subtracted from the response obtained for the spiked samples.

Table 3. %ME, $\%R_{SPE}$ and $\%R_{apparent}$ obtained when 100 mL of influent wastewater spiked at 5000 ng/L were percolated in both Oasis MCX and Oasis MAX sorbents.

	Oasis MCX			**Oasis MAX**		
	%ME	$\%R_{SPE}$	$\%R_{apparent}$	%ME	$\%R_{SPE}$	$\%R_{apparent}$
BTRs						
BTR	-21	77	61	-22	77	59
4TTR	-11	96	85	-19	82	66
5TTR	0	87	86	-7	84	77
ClBTR	-13	67	58	-21	64	50
XTR	0	89	92	0	82	80
BTs						
BT	24	85	105	0	52	52
NH_2BT	-25	86	64	-18	40	40
OHBT	-32	58	40	-29	90	63
MeSBT	-22	66	52	-26	86	64

%RSD (n=3) <16%

The $\%R_{SPE}$ recoveries reported in Table 3 were similar in both sorbents except for certain differences (also observed in ultrapure water), such as the case of NH_2BT, which showed less retention (40%) in the Oasis MAX cartridge than in the Oasis MCX sorbent (86%).

The %ME values obtained for both sorbents were also similar. The %ME obtained for the Oasis MCX sorbent ranged from -32% to 24%, while values obtained for the Oasis MAX sorbent were between -29% and 0%. It can be seen that signal suppression predominates over enhancement, as only BT presented positive values of %ME. In order to observe the effect of the MeOH washing volume on the %ME, the second wash step of the SPE procedure was performed with 5 mL of pure MeOH instead of 2 mL. For the Oasis MCX sorbent, %ME values ranged between -28% and 10%, indicating that no significant decrease in %ME is observed when increasing the volume of MeOH wash. Furthermore, NH_2BT and BT were collected in the wash fraction. Likewise, in the case of the Oasis MAX sorbent, the differences in the %ME were not significant when increasing the volume of MeOH, so the change does not compensate the fact that more analytes were also rinsed in the wash fraction. It can be observed that the first millilitres of MeOH are the most important in eluting hydrophobic interferences and cleaning the matrix. Thus, for both sorbents, it was decided to use 2 mL in the second wash with MeOH. The recoveries obtained for influent wastewaters corresponding to the whole method ($\%R_{apparent}$) are also detailed in Table 3. As can be seen, $\%R_{apparent}$ values are similar to or slightly lower than the $\%R_{SPE}$ values, as expected since %ME was low.

Subsequently, the method was evaluated in river water and effluent wastewaters using both sorbents. The results obtained are detailed in Table 4. The %ME values obtained for 250 mL of river water and effluent wastewater samples were lower than those obtained for influent wastewater samples, even when a higher volume of sample was employed. For the Oasis MCX sorbent, %ME values ranged from -4% to 20% and -11% to 11% for river water and effluent wastewater samples, respectively. In the case of the Oasis MAX sorbent, %ME values ranged between -19% and 16% and between -25% and 6% for river

and effluent wastewater samples, respectively. It can be seen that the %ME obtained was very low for this type of matrices when performing the washing steps proposed in the SPE procedure. It was also noticeable that, in contrary to influent samples, river samples showed enhancement in most of the cases. In general, a %ME below 20% can be considered not significant, hence, the values obtained in this study in environmental waters is quite acceptable for these types of matrices.

The %ME found for these compounds in complex environmental waters was lower than or similar to several studies. Herrero et al. [20] placed a Florisil cartridge (500 mg) in series after the Oasis HLB cartridge to obtain clearer extracts. They reported comparable values (from -29% to 4%) to those obtained here, but as they needed the additional Florisil cartridge while the present procedure uses only the mixed-mode sorbent, the present study shows the advantage of a simplified SPE procedure. It must be pointed out that in the same study [20], the performance of the Oasis MAX in effluent wastewater was compared with the Oasis HLB, but results were unsatisfactory because the procedure used was not optimized. In the study by Carpinteiro et al. [19], the Oasis MAX sorbent was used, but focusing on retaining ionic interferences of the matrix rather than the actual analytes. In their method, the analytes were collected with a mixture of MeOH and acetone, while acidic interferences remained retained in the sorbent through ionic interactions. Using this procedure, they obtained clearer extracts, reporting no ME for river samples and a small decrease in the response for effluent wastewater samples. Nevertheless, influent wastewater samples and industrial waters showed high ME (up to around -50%) and standard addition or matrix-matched calibration was suggested.

For river water and effluent wastewater samples, the $\%R_{apparent}$ were from 77% to 105% and between 54% and 92%, respectively, when the Oasis MCX sorbent was used. In the case of the Oasis MAX sorbent, $\%R_{apparent}$ values for river and effluent wastewater samples were from 65% to 93% and between 44% and 87%. As the matrix has a low effect on the response for these samples, the $\%R_{apparent}$ were almost the same recoveries obtained for the $\%R_{SPE}$.

Up to this point, both sorbents exhibit the capacity to interact with the analytes, allowing washing with pure MeOH to achieve low %ME, even when the compounds are not ionic per se. Hence, both sorbents were further compared during the method validation in order to evaluate whether there was any difference with regard to their validation parameters.

3.3 Method validation

The two methods based on the Oasis MCX and Oasis MAX sorbents were validated in the environmental waters using the SPE procedure detailed in Section 2.3 for each case. As some of the analytes (such as 4TTR and 5TTR) were present in the blanks at relatively high concentrations, the use of matrix-matched calibration curves for quantification was not possible. Considering that $\%R_{apparent}$ values were satisfactory (Table 4) and that the repeatability of the method with both sorbents was great, quantification in the environmental waters was propo-

Table 4. %ME and $\%R_{apparent}$ obtained when 250 mL of river and effluent wastewater samples spiked at 2000 ng/L were percolated in both Oasis MCX and Oasis MAX sorbents.

	Oasis MCX				**Oasis MAX**			
	River		Effluent		River		Effluent	
	%ME	$\%R_{apparent}$	%ME	$\%R_{apparent}$	%ME	$\%R_{apparent}$	%ME	$\%R_{apparent}$
BTRs								
BTR	14	96	-9	67	5	75	-2	90
4TTR	12	103	-6	74	4	87	-15	82
5TTR	13	103	-1	92	11	89	-14	79
ClBTR	14	87	-6	61	5	77	-22	66
XTR	20	105	11	90	16	92	2	83
BTs								
BT	12	92	9	72	8	65	6	52
NH_2BT	3	103	-7	86	-6	65	-10	44
OHBT	19	77	-11	54	2	72	-23	68
MeSBT	-4	83	-2	67	-19	93	-25	69

%RSD (n=3) <20%

sed using an external calibration method, taking into account the $\%R_{apparent}$.

As an example, the $\%R_{apparent}$ values obtained for effluent samples during the validation (evaluated for two levels of concentration, 100 ng/L and 1,000 ng/L) will be discussed in more detail. For the Oasis MCX sorbent, the $\%R_{apparent}$ obtained when the sample was spiked at 100 ng/L were from 69% to 97%, except for ClBTR (51%), while values obtained at 1,000 ng/L were from 52% to 89%. In the case of the Oasis MAX sorbent, the $\%R_{apparent}$ at low level ranged between 78% and 111%, except for ClBTR (51%) and, at the high level, were from 67% to 91%, except for BT (47%). It can be seen that results were very similar to those obtained when spiking 2,000 ng/L during optimization (Table 4). Most of the $\%R_{apparent}$ obtained were satisfactory, except for the few compounds that displayed less retention on the sorbents. The $\%R_{apparent}$ obtained for river samples and influent samples were also in agreement with the values obtained during optimization, therefore, they were considered reliable for quantification purposes. This proved to be satisfactory when evaluating the $\%R_{relative}$ values (spiking a fresh volume of effluent wastewater sample and analysing it as unknown to compare the concentration obtained with the expected value) for two different levels of concentration, 100 ng/L and 1,000 ng/L, obtaining values from 85% to 110%.

For both sorbents, the repeatability and detection and quantification limits of the method were evaluated. Repeatability was also evaluated for two levels of concentration (100 ng/L and 1,000 ng/L) and was expressed as the relative standard deviation (%RSD, n=5). Only the higher level was considered for those analytes that were present in the blanks. It was observed that repeatability was excellent, as it was lower than 19% in every case. As example for effluent samples and for the lower concentration, values ranged between 2% and 19% and, for the higher concentration, the values were between 3% and 13%. Similar results were observed for both sorbents and the different matrices with regard to repeatability.

The method limits (MDLs and MQLs) were estimated from the instrumental limits (ILODs and ILOQs, referred to in Section 3.1) and the $\%R_{apparent}$ values. In more detail, ILODs and ILOQs were

multiplied by 100% and divided by the $\%R_{apparent}$ values. The result of this calculation was subsequently divided by the volume of the sample and the final result was expressed in ng/L. For both sorbents, results were very similar, observing that, for river samples, MDLs ranged from 2 to 60 ng/L and, for effluent and influent wastewaters, they were between 3 and 50 ng/L and between 10 and 225 ng/L, respectively. MQLs ranged from 5 to 155 ng/L for river samples and from 6 to 190 ng/L and from 15 to 565 ng/L for effluent and influent wastewater samples, respectively. In every case, it was found that the higher limits corresponded to BT, OHBT and MeSBT. These results are similar to those found in the literature, even when the detectors used were not the same. Using their LC-MS/MS method, Carpinteiro et al. [19] found LOQs that ranged from 2 to 286 ng/L in river water and wastewaters, also finding the higher limits for BT, OHBT and MeSBT. In the LC-MS/MS method developed by Loi et al. [14], LOQs between 6 and 1072 ng/L were reported for their selected group of BTRs and BTs in secondary wastewater, finding higher values for BT and OHBT. Jover et al. [22] found LOQs between 31 and 99 ng/L for river samples and between 62 and 198 ng/L for wastewater samples, when using TOF-MS detection. Thus, obtained values were in the same order of magnitude of several studies summarized in the literature [10]. During this section, differences between both sorbents were not substantial; therefore, their performance will be compared during the application of the method.

3.4 Application to environmental samples

Three different samples of river water, effluent and influent wastewaters were analyzed in both sorbents in order to compare the results obtained. The range of concentrations found is shown in Table 5. It can be seen that the results obtained did not show considerable differences between the sorbents. Almost all of the compounds were found at significant levels in the analysed samples. BTR, 4TTR and 5TTR presented the highest concentrations of all of the compounds, while ClBTR and MeSBT were found in concentrations below the LOQs.

In influent wastewater, BTR, 4TTR and 5TTR showed the highest concentrations of all of the analytes (up to 1980 ng/L for BTR, Table 5). Lower

concentrations were found for BT (up to 767 ng/L) and OHBT (up to 450 ng/L) and low concentrations of XTR and NH_2BT could be successfully quantified. The results found for this type of sample are in line with the concentrations found in other studies [10]. Herrero et al. [20] found concentrations of BTR, 4TTR and 5TTR ranging between 392 and 2212 ng/L in influent wastewaters collected from the same Tarragona sewage treatment plant. Concentrations of OHBT (199 ng/L) and NH_2BT (10 ng/L) were also around the same order of magnitude, while XTR and BT levels found were lower than their LOQ. In influent wastewater samples collected also from a sewage treatment plant in Spain, Carpinteiro et al. [19] found average levels of BTR, TTR, OHBT and BT of 2,470, 2,100, 150 and 830 ng/L, respectively.

In the case of effluent wastewaters, it was found that concentrations of BTR, 4TTR and 5TTR were very similar to those obtained for influent wastewaters. In effluent wastewaters, concentrations were slightly higher, probably because samples were collected randomly on different days. BT, NH_2BT and OHBT showed concentrations lower than those obtained in influent wastewaters, suggesting partial removal or degradation, as expected after the treatment process. Some of these results are in agreement with the literature, such as the study by Asimakopoulos et al. [12], in which the removal efficiencies of BTRs and BTs were studied in a treatment plant in Greece. They found low removal rates for BTR and TTR (4TTR and 5TTR), ranging from 25% to 68%, and high removal rates for OHBT. However, they highlighted that there is a discrepancy in the literature concerning the removal efficiencies of these analytes, especially in the case of BTs, so further comparison is not reliable. In the case of river waters, relatively high concentrations of BT (up to 286 ng/L) were found compared to other compounds. Herrero et al. [20] reported a concentration of 60 ng/L for the Ebre River. The range of concentrations found for this compound in rivers in Spain was between 30 and 200 ng/L [10]. Lower concentrations of the rest of the compounds were found, ranging from 24 to 101 ng/L. It can be seen that these analytes are present in rivers, due to anthropological sources, such as industrial spills or incomplete removal in treatment plants.

Table 5. Range of concentrations (ng/L) obtained when river, effluent and influent wastewater samples were analysed using the validated method, based on mixed-mode SPE followed by LC-HRMS.

	River (ng/L)		**Effluent (ng/L)**		**Influent (ng/L)**	
	Oasis MCX	Oasis MAX	Oasis MCX	Oasis MAX	Oasis MCX	Oasis MAX
BTRs						
BTR	24 - 27	< LOQ - 30	544 - 2342	421 - 2067	954 - 1978	936 - 1980
4TTR	< LOQ - 15	< LOQ - 18	994 - 1697	666 - 1308	743 - 1236	743 - 1210
5TTR	28 - 43	30 - 47	971 - 1881	768 - 1610	687 - 1253	694 - 1043
ClBTR	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 10
XTR	< LOQ	< LOQ	< LOQ - 162	< LOQ - 128	< LOQ	< LOQ - 58
BTs						
BT	176 - 286	218 - 221	243 - 272	265 - 358	286 – 431	474 - 767
NH_2BT	31 - 32	41 - 43	30 - 34	55 - 59	70 - 71	155 - 160
OHBT	86 - 94	91 - 101	123 - 182	92 - 136	410 - 450	347 - 408
MeSBT	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

The applicability of both methods was confirmed, as several compounds were found and quantified in the samples analysed.

4 Conclusions

The mixed-mode strong cation-exchange and anion-exchange sorbents can successfully retain the group of BTRs and BTs studied, with enough strength to allow washing with pure MeOH in order to develop a more selective extraction method. This was possible because the analytes and the ionic groups of the sorbent establish ionic interactions, which seem to be stabilized by the resonance along both aromatic rings of the compounds capable of delocalizing electron densities. The strong character of the groups attached to the sorbents might have induced partial charges in the analytes promoting ion-dipole interactions. In contrary, the selected BSAs were always rinsed in the washing fractions probably because only hydrophobic interactions are involved in their retention. For this reason, these analytes were not further subject and only BTRs and BTs were more deeply studied. These types of interpretations are important in the development and evaluation of the mixed-mode sorbents used in analytical applications as they help to understand their capabilities to retain not only ionic analytes but also compounds that may develop partial charges.

It has been demonstrated for the first time that mixed-mode cation-exchange and anion-exchange sorbents can retain BTRs and BTs trough ionic interactions even when they are neutral under working conditions, confirming that these sorbents not only work with pure ionic analytes but also neutral ones that are highly polarizable.

No substantial differences were observed in their performance when both sorbents were compared in river water and effluent and influent wastewater samples. Thanks to the possibility of including a pure MeOH washing step, the %ME obtained was around 20% or lower for wastewater samples and was practically negligible for river samples. The developed method using either Oasis MCX or Oasis MAX followed by LC-HRMS provided better results compared to other studies found in the literature.

Acknowledgments

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Supplementary Data

Table S1. Detection limits and quantification limits obtained for the different matrices during validation of the method.

	River				Effluent				Influent			
	MDL (ng/L)		MQL (ng/L)		LOD (ng/L)		LOQ (ng/L)		LOD (ng/L)		LOQ (ng/L)	
	MCX	MAX	MCX	MAX	MCX	MAX	MCX	MAX	MCX	MAX	MCX	MAX
BTRs												
BTR	8	11	17	21	12	10	23	20	24	28	48	57
4TTR	12	14	12	14	16	15	16	15	35	45	35	45
5TTR	12	13	12	13	16	14	16	14	36	40	36	40
ClBTR	2	3	5	5	4	4	8	8	8	9	16	18
XTR	15	17	15	17	18	17	18	17	44	51	44	51
BTs												
BT	43	61	108	153	41	40	103	99	107	226	268	564
NH2BT	3	5	6	9	3	8	6	15	8	19	16	38
OHBT	47	50	52	55	46	32	51	36	184	156	205	173
MeSBT	24	21	120	107	38	31	192	156	62	80	309	401

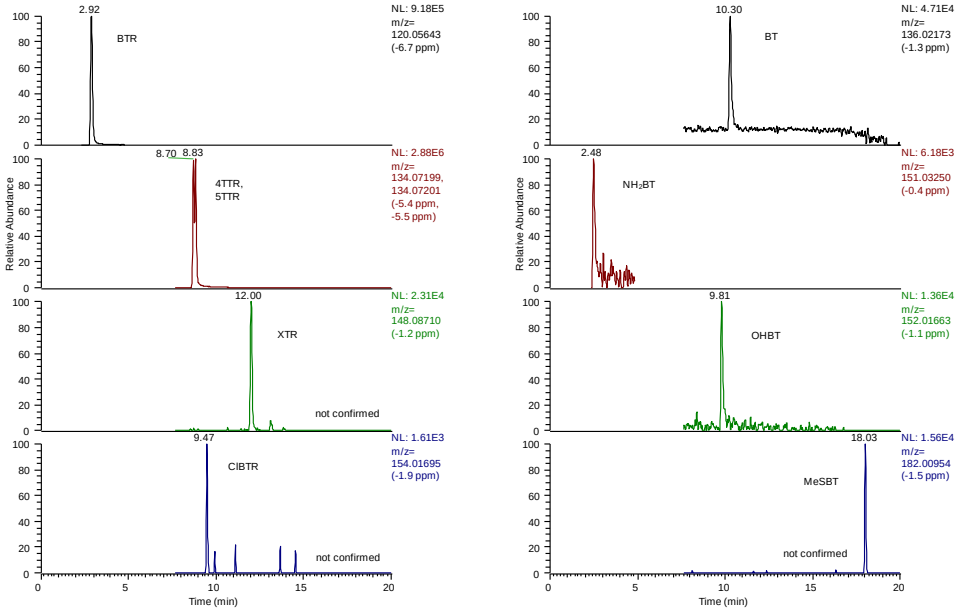

Figure S1. Chromatogram of an effluent wastewater sample when analyzed under optimum conditions using the Oasis MAX sorbent.

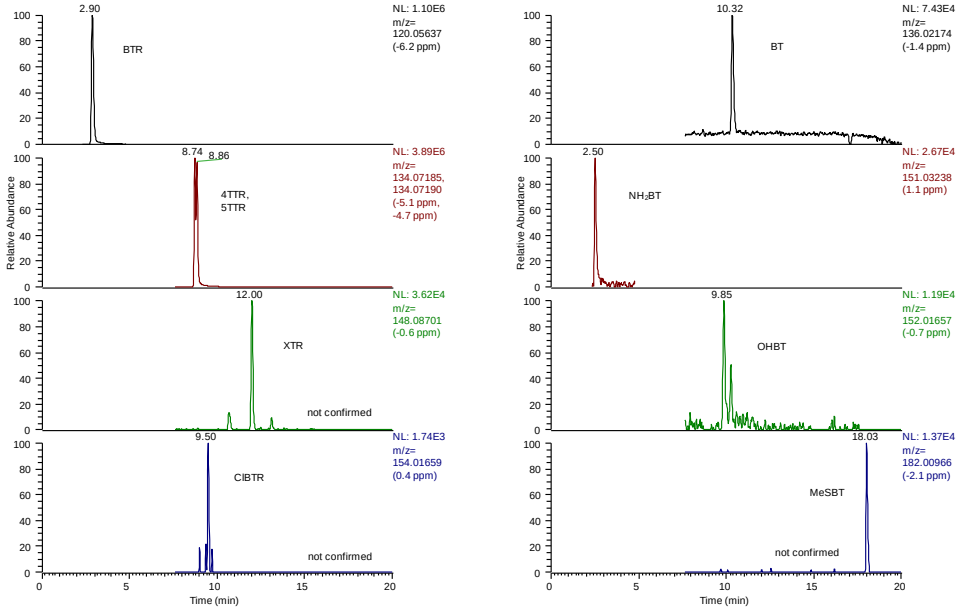

Figure S2. Chromatogram of an effluent wastewater sample when analyzed under optimum conditions using the Oasis MCX sorbent.

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
Daniela Salas Acosta

3.2.2. Combining cationic and anionic mixed-mode sorbents in a single cartridge to extract basic and acidic pharmaceuticals simultaneously from environmental waters

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COMBINING CATIONIC AND ANIONIC MIXED-MODE SORBENTS IN A SINGLE CARTRIDGE TO EXTRACT BASIC AND ACIDIC PHARMACEUTICALS SIMULTANEOUSLY FROM ENVIRONMENTAL WATERS

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Abstract

The success of mixed-mode solid-phase extraction sorbents as cationic or anionic exchangers is currently limited to extracting the selected basic or acidic analytes separately if a washing step is included to remove neutral compounds. The aim of the present study is to broaden the applications of these sorbents to extract both basic and acidic compounds simultaneously by combining the sorbents in a single cartridge and developing a simplified extraction procedure. Four different cartridges containing negative and positive charges in the same configuration were evaluated and compared to extract a group of basic, neutral and acidic pharmaceuticals selected as model compounds. The solid-phase extraction conditions, such as the pH of the loading and eluting steps, were carefully optimized and thoroughly discussed, and special attention was paid to the pK_a values of the analytes and the functional groups attached to the sorbents. The four different cartridges were capable of simultaneously retaining basic and acidic pharmaceuticals through ionic interactions, allowing the introduction of a washing step with 15 mL methanol to eliminate interferences retained by hydrophobic interactions. Moreover, the retention behaviour of the selected analytes on the sorbents was in line with their pK_a values, confirming the presence of ionic interactions as the predominant retention mechanisms. Using the best combination of sorbents, a method was developed, validated and further applied to environmental waters, achieving similar results to those reported in more time-consuming methods. The method is promising in terms of being tested on other basic and acidic compounds and other matrices of high complexity.

Keywords: *cationic mixed-mode solid-phase extraction; anionic mixed-mode solid-phase extraction; sorbent combination; high-resolution mass spectrometry; environmental waters.*

1 Introduction

Solid-phase extraction (SPE) remains the most commonly used sample preparation technique for liquid samples in several analytical methods. Its wide acceptance is the result of the great advantages that it provides, such as the enrichment of the analytes with high recoveries and the enhanced selectivity thanks to the availability of different types of sorbents [1]. SPE is often combined with chromatographic techniques such as liquid chromatography (LC) and gas chromatography (GC) coupled with mass spectrometry (MS) detection, obtaining methods with high sensitivity and selectivity to determine several target compounds in complex matrices at trace levels [2-5].

Among the different sorbents available for SPE, mixed-mode sorbents combine a polymeric structure with one type of ionic functional groups (cationic or anionic) in a single cartridge, giving them the capability of retaining compounds through reversed-phase and ion-exchange interactions [6]. They were developed to promote selectivity for ionic compounds, broadening the groups of analytes that can be retained by a single sorbent [7-9]. The most important feature of mixed-mode sorbents is the possibility of including a washing step with organic solvents in the SPE procedure, which allows the elimination of interferences retained by hydrophobic interactions. In several studies, the potential of these sorbents has been proven to reduce the matrix effect (ME) in LC-MS based methods, by eliminating interferences during washing steps in the SPE procedure [10-13].

There are four types of mixed-mode sorbents depending on the functional groups attached to the polymer particles: strong or weak cationic-exchangers (SCX or WCX) and strong or weak anionic exchangers (SAX or WAX). The sorbents with strong properties include functional groups that are charged in the entire pH range (such as sulfonic acid or quaternary amine groups), while those with weak properties have groups with a more reduced working pH range depending on their pK_a values (carboxylic acid or tertiary or secondary amine groups). When working with any of these sorbents, the selection of the SPE conditions is very important, as parameters such as pH or type of clean-up and elution solvent have a great impact on the performance of the sorbents [6]. By fine-tuning the

SPE protocol, these sorbents can extract an extensive range of compounds with different properties (non-polar, polar or ionic) or they can be selective towards ionic compounds if washing steps are applied.

Successful applications of mixed-mode sorbents commercially available or prepared in-house with strong or weak properties have been reported, describing the extraction of different groups of compounds from complex matrices [14-19]. Analytes such as pharmaceuticals, drugs of abuse and compounds of biological interest have been extracted from environmental waters, foodstuff and biologic fluids [20-23]. In most of the studies published to date, the potential of mixed-mode sorbents is limited to basic or acidic analytes depending on the type of ion-exchanger selected. Only a few studies have explored the result of combining both types of sorbents to extract a whole list of target compounds with both basic and acidic properties. Lavén et al. [24] developed an SPE procedure in which a SCX sorbent was placed in tandem with an SAX sorbent to extract 15 basic, neutral and acidic pharmaceuticals simultaneously. More recently, Deeb et al. [25] proved that a tandem combination of SCX and SAX sorbents gave the highest recoveries in ultrapure water when compared to several SPE sorbents. However, the procedures of these methods are more complicated than those using a single cartridge. The aim of the present study is to broaden the use of mixed-mode sorbents to extract both basic and acidic groups of compounds simultaneously by combining for the first time mixed-mode sorbents with strong or weak cationic and anionic properties to obtain single SPE cartridges with balanced positive and negative charges. When combining the sorbents in a single cartridge, the SPE procedure is simplified as long as the SPE conditions are correctly selected. With this in mind, a systematic evaluation was performed of combinations of the four types of mixed-mode sorbents (SCX, WCX, SAX and WAX) by pairs with opposite charges for the selective extraction of ionizable pharmaceuticals. The effect of having strong or weak properties in the sorbents was observed, as well as the effect of changing the pH and other parameters on the performance of the extraction. Subsequently, the best combination of sorbents was evaluated in environmental waters in terms of recoveries and ME using LC-high resolution mass spectrometry (HRMS).

2 Experimental

2.1. Reagents and standards

The pharmaceuticals or metabolites with basic properties, atenolol (ATE), ranitidine (RAN), trimethoprim (TRI), metoprolol (MET) and propranolol (PROP); as well as the neutral pharmaceuticals, caffeine (CAFF), antipyrine (ANTI) and carbamazepine (CBZ); and those with acidic properties, salicylic (SAL AC) and clofibric acid (CLO AC), fenoprofen (FEN), diclofenac (DICLO) and ibuprofen (IBP), were purchased as pure standards from Sigma-Aldrich (St. Louis, MO, USA). The structures, pK_a values and exact masses of all of the analytes are shown in Table S1. Solid standards were dissolved in methanol (MeOH) to prepare stock solutions of 1 mg/mL which were stored at -20°C. Working solutions with the mixture of all the pharmaceuticals were prepared in ultrapure water every week and stored at 4°C.

Ultra-gradient HPLC grade MeOH and acetonitrile (ACN) were obtained from J.T. Baker (Deventer, the Netherlands), while ultrapure water was obtained from a water purification system (Veolia, Sant Cugat del Vallès, Spain). Acetic acid (CH_3COOH) and formic acid (HCOOH) were purchased from SDS (Peypin, France) and Sigma-Aldrich, respectively. Ammonium hydroxide (NH_4OH) was obtained from Panreac (Barcelona, Spain).

2.2. Sampling

River water samples were collected from the Ebre River in Catalonia, while influent and effluent wastewater samples were collected from sewage treatment plants located in Tarragona and Reus. Both treatment plants include primary and secondary treatments in their processes. Once the samples were collected in pre-cleaned bottles, they were stored at -20°C until the day of analysis. Before any SPE procedure, the samples were filtered through a 1.2 μm glass-fibre membrane filter (Fisherbrand, Loughborough, UK) and then through a 0.22 μm nylon membrane filter (Scharlab, Barcelona, Spain).

2.3. Solid-phase extraction procedure

Four different 100 mg cartridges containing combinations of individual sorbents with different functional groups (Table 1) were evaluated to extract basic and acidic pharmaceuticals

simultaneously. The amount in grams of each individual sorbent used for each cartridge was established to obtain balanced cationic and anionic moieties. The configuration that gave the best results was the strong cationic/strong anionic one (SCX/SAX). Therefore, the protocol was transferred to 500 mg cartridges (110 mg of SCX and 390 mg of SAX) of this combination to extract the environmental waters. These cartridges were conditioned with 10 mL of MeOH, followed by 10 mL of ultrapure water adjusted to pH 7. The selected loading volumes were 100 mL for river water and effluent wastewater samples and 50 mL for influent wastewater samples, which were adjusted to pH 7. In each case, pH was adjusted using NH_4OH or HCOOH. After loading the samples, a washing step was introduced consisting of 15 mL of MeOH. The elution was performed in two subsequent steps: 1) 5 mL of a 10% HCOOH in MeOH solution; and 2) 5 mL of a 5% NH_4OH in MeOH solution. Both fractions were collected in the same vial and the extract was evaporated to dryness using a centrifugal evaporator miVac Duo (Genevac, Zaragoza, Spain) and later reconstituted with 1 mL of ultrapure water for river water samples and 2 mL for effluent and influent wastewater samples.

Table 1. Configurations of the four cartridges prepared from the individual mixed-mode sorbents.

Individual sorbent	Ionic functional group	meq/g	Configuration	mg	meq
SCX	sulfonic acid	1	1) SCX/SAX	22 SCX	0.0220 SCX
				78 SAX	0.0195 SAX
SAX	dimethylbutylamine	0.25	2) SAX/WCX	76 SAX	0.0190 SAX
				24 WCX	0.0180 WCX
WCX	carboxylic acid	0.75	3) SCX/WAX	38 SCX	0.0380 SCX
				62 WAX	0.0372 WAX
WAX	piperazine	0.6	4) WCX/WAX	44 WCX	0.0330 WCX
				56 WAX	0.0336 WAX

2.4. LC-HRMS

A Thermo Scientific Accela 1250 UHPLC system (Bremen, Germany) equipped with an Accela Autosampler automatic injector and an Accela 1250 pump was coupled with a Thermo Scientific Exactive Orbitrap™ mass spectrometer for the chromatographic analysis. The mass spectrometer worked with a heated electrospray ionization (HESI) source and a higher energy collisional dissociation (HCD) cell. The chromatographic column used was the Ascentis Express C_{18} (100 mm x 2.1 mm i.d., 2.7 μm particle size) supplied by Supelco (Sigma-Aldrich), and the mobile phase was a mixture of solvent A (0.5% CH_3COOH in H_2O) and solvent B (MeOH). The column was kept at 25°C and the mobile phase was pumped at 400 μL/min. The injection volume used was 25 μL and the tray of vials inside the automatic injector was kept at 10°C. The optimal gradient profile started with 2% of solvent B which was increased to 30% within 6 min and then increased again to 80% within 6 min and held for a further 1 min. After this, solvent B was increased to 100% within 2 min and left isocratic for 3 min and later returned to the initial conditions within 2 min.

In the ion source, basic pharmaceuticals (Table S1) were ionized in the positive mode using the following optimal parameters: spray voltage, 2 kV; skimmer voltage, 25 V; capillary voltage, 40 V and tube lens voltage, 80 V. In the case of acidic pharmaceuticals, the negative ionization mode was used and the optimal parameters were: spray voltage, 3.5 kV; skimmer voltage, -15 V; capillary voltage, -15 V and tube lens voltage, -80 V. Gas flow rates and temperatures were the same for both ionization modes: sheath gas, 40 AU (adimensional units); auxiliary gas, 5 AU; heater and capillary temperature, 350°C; and probe position adjustment: side to side, 0, vertical C and micrometer, 0.5.

Four time windows were used to acquire the data: the first and third (0 to 7.5 min, and 9.5 to 11 min) were set in positive mode, the second (7.5 to 9.5 min) in both modes and the last (11 to 20 min) in negative mode. In all of the windows, two scan events were used for each ionization mode, corresponding to a full scan (at 50,000 FWHM with 250 ms of injection time), which was alternated with a fragmentation scan (at 10,000 FWHM with 50 ms of injection time). Because the second window operated in both

positive and negative modes, four scan events were used. The optimal voltage in the HCD cell selected in all the fragmentation scans was 25 eV. For quantification, the response of the molecular ions was used and, for confirmation, the presence of the most abundant fragment ions and the corresponding ion ratios were considered.

3 Results and discussion

3.1. Optimization of LC-HRMS conditions

There are several studies in the literature describing the chromatographic separation of the group of pharmaceuticals selected in the present study, using several brands of stationary phases with different characteristics and different conditions for the mobile phase and the rest of the chromatographic parameters. The use of a C_{18} stationary phase is quite common and the dimensions of the column are normally selected according to availability or the time of analysis desired [26-28]. For this study, the Ascentis Express C_{18} (100 mm x 2.1 mm i.d., 2.7 µm particle size) was initially selected and compared to the Ascentis Amide (100 mm x 2.1 mm i.d., 2.7 µm particle size) which has proven to offer better retention for polar compounds [29]. The mobile phase was optimized with respect to the organic solvent (ACN or MeOH) and the type of acid added to the aqueous phase (HCOOH or CH_3COOH). The optimal pH for the separation of the analytes was 2.8. Tests regarding the flow-rate and the temperature were also performed to improve the separation.

The optimal parameters for obtaining the best chromatographic separation are detailed in Section 2.4. As relevant observations, it can be said that CH_3COOH was chosen over HCOOH because it offered better ionization for FEN and IBP in the ion source, achieving a higher response for these compounds, which showed lower signal when compared with the rest of the analytes. Both organic solvents, ACN and MeOH, were tested on both stationary phases achieving different selectivity with advantages and drawbacks in each case. The best results were obtained using ACN in the amide phase and MeOH in the C_{18} phase. However, in the amide phase, the first eluting compounds eluted near the void volume, while the C_{18} phase showed higher retention for these compounds with a

satisfactory time of analysis. For this reason, the C_{18} column was selected, using MeOH as the organic solvent of the mobile phase.

With respect to the HRMS instrument, the response of the molecular ions [M+H]+ or [M-H]- of all the analytes was monitored by direct injection of 1,000 μg/L solutions with and without a flow of the mobile phase in order to observe changes in the signal when varying the ionization parameters. The spray, capillary, tube lens and skimmer voltages, as well as the sheath and auxiliary gas flow rates and the capillary and heater temperatures, were optimized as a compromise of the highest response for all of the compounds. Moreover, the position of the probe was optimized horizontally (-1 to 1), vertically (A, B, C or D) and in the z axis using the micrometer. As expected, it was observed that basic and neutral pharmaceuticals (ATE, RAN, TRI, CAFF, MET, ANTI, PROP and CBZ) showed the highest response in the positive mode, in contrast to acidic pharmaceuticals (SAL AC, CLO AC, FEN, DICLO and IBP), which were better ionized in the negative mode. The optimal values obtained for the voltages, gas flow rates, temperatures and probe position mentioned above are indicated in Section 2.4.

For the optimization of the fragmentation, 1,000 μg/L individual solutions of each analyte were directly infused in the spectrometer and the response of the fragments was monitored while the voltage in the HCD cell was increased, aiming to obtain the highest response. For the selected group of pharmaceuticals, a single fragmentation voltage (25 eV) was selected that was suitable for obtaining a satisfactory response of all the fragments of all the analytes. The exact mass of the molecular ions and the selected fragments for each analyte are shown in Table S1 and they are in line with the literature [30-33]. Fragmentation was troublesome in the case of ANTI because the molecule yielded several fragments with low response, but finally the one selected for confirmation gave a satisfactory ion ratio. The fragments that displayed the highest response were considered to determine the instrumental limits of detection (ILODs), which were the concentrations at which the peak corresponding to the fragment showed a signal to noise ratio (S/N) of 3, as widely accepted, or the signal was higher than $1x10^3$ for the analytes for which noise was not observed.

The instrumental limit of quantification (ILOQ) was the concentration corresponding to the first point of the calibration curve. The observed ILODs ranged between 0.05 and 2.5 μg/L, while the ILOQs ranged between 0.2 and 2.5 μg/L, with FEN, DICLO and IBP being the analytes with higher limits, due to their lower response. Linearity was evaluated from 0.2 to 1,000 μg/L, with lack of linearity being observed for the entire range. Therefore, low level and high level calibration curves were constructed for most of the analytes. Each analyte showed different ranges of linearity but, in general, low level calibration curves were between 0.2 and 100 μg/L, while high level calibration curves were between 25 and 1,000 μg/L.

3.2. Solid-phase extraction

3.2.1. Behaviour of the four cationic/anionic combinations

Individual sorbents with four different functionalities (SCX, WCX, SAX and WAX) were combined by pairs with opposite charges in single cartridges so that the two charges were balanced, so each group had the same ionic exchange capacity. As can be seen in Table 1, the result was four different types of cartridges with cationic/anionic functionalities and strong/strong, strong/weak or weak/weak properties.

The extraction of the 13 selected pharmaceuticals was evaluated in the four different 100 mg cartridges, using a SPE procedure designed to retain basic and acidic pharmaceuticals simultaneously while washing away neutral interferences. Careful attention was paid to the pH values of the loading and the elution step, taking into account the pK_a of the functional groups of the sorbents and the pK_a of the analytes, because, when loading the sample, both the functional groups of the sorbents and the analytes must be in their ionic form in order to establish ionic interactions [6, 34, 35]. In contrast, in the elution step, either the functional groups of the sorbents and/or the analytes must be in their neutral form to disrupt retention and favor the elution of the analytes.

For instance, a sample can be loaded in the SAX/SCX configuration using a pH value between 5 and 8 (Figure 1) because basic analytes are charged up to ~pH 8 and acidic analytes are charged from ~pH 3 or 5 (see pK_a values in Table S1). The pK_a

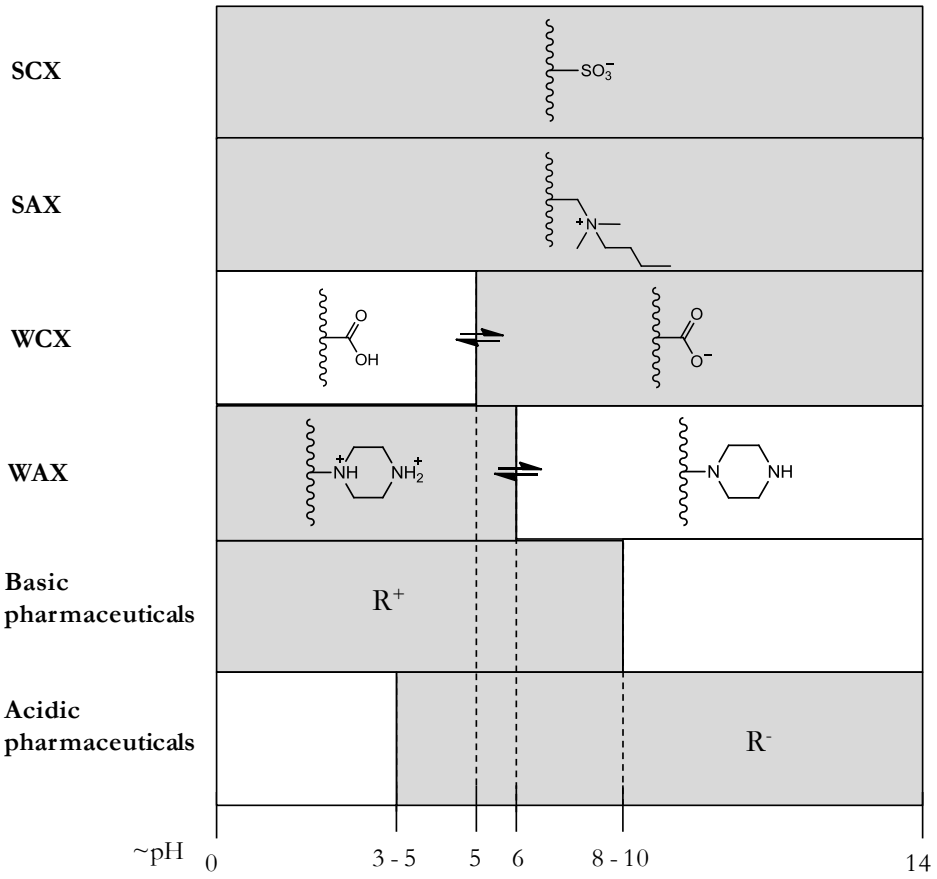

Figure 1. Charge state of functional groups of the sorbents and the analytes along the pH range (indicated in grey when charge state is ionic).

values of the moieties of the sorbents were not taken into account in this case because strong functionalities are always charged throughout the pH range. Therefore, the pH value for the elution was selected such that the analytes are converted into their neutral form. Acidic pharmaceuticals were eluted using an acidic solution while basic pharmaceuticals were collected using a basic solution.

When weak moieties are included in the configuration of the cartridges, the pK_a of the functional groups attached to the sorbents must be considered. In the case of the SCX/WAX configuration, the pK_a of the piperazine group attached to the polymer (WAX) is ~6, as stated by

the manufacturer. Therefore, the optimal loading pH range is between 5 and 6 (Figure 1), considering the pK_a values of the analytes and the sorbents. Furthermore, all of the analytes should elute in a single fraction when using a basic solution because basic pharmaceuticals would convert into their neutral form, as well as the piperazine groups in the polymer, disrupting their interactions with the acidic pharmaceuticals.

As a result of the previous considerations, the SPE procedure initially used for the four configurations is described below, considered to be the best conditions according to the theory. The cartridges were conditioned with 10 mL of MeOH followed by 10 mL of ultrapure water adjusted to pH 5 using HCOOH. A volume of 50 mL of ultrapure water adjusted to pH 5 spiked with a mixture of the analytes was percolated through the cartridges. The washing step introduced consisted of two fractions of 1 mL of MeOH. Finally, the elution step was performed in two steps: 1) 5 mL of a 5% HCOOH solution and, 2) 5 mL of a 5% NH_4OH solution for all the cartridges, except for the SCX/WAX configuration, for which the elution steps were interchanged (the basic elution was performed before the acidic elution).

The results obtained in the SPE evaluation were fully in line with the expected behavior according to the pK_a values and are shown in Table 2. All of the analytes were retained by the cartridges during the loading step, which was expected, as the combinations of the sorbents should have the capability of retaining basic, acidic and neutral compounds, thanks to the polymeric backbone and the functional groups of the sorbents. During the washing step, only CAFF, ANTI and CBZ were completely rinsed, which is in line with their pK_a values, as they are neutral compounds. Their retention in cation exchangers has been proven [36], behavior that could be explained by their ability to develop partial charges through electron delocalization. However, in a cartridge in which negative charges are coexisting with positive charges, this delocalization might be compromised and so the analytes end up behaving as neutral compounds rather than weak bases.

The rest of the pharmaceuticals were rinsed during the elution

Table 2. Recoveries (%) obtained when 50 mL of ultrapure water adjusted at pH 5 were percolated thourgh the four cartridge combinations.

		SCX/SAX				SAX/WCX					SCX/WAX					WCX/WAX				
	Analyte	w1	w2	e_a	e_b	Σ	w1	w2	e_a	e_b	Σ	w1	w2	e_b	e_a	Σ	w1	w2	e_a	e_b	Σ
basic	ATE			76	76				85		85			81		81			82		82
	RAN			79	79				64		64			80		80			56		56
	TRI			82	82				85		85			81		81			85		85
	MET			76	76				85		85			80		80		2	83		85
	PROP			82	82				92		92			86		86			91		91
neutral	CAFF	76	19			94	88	11			100	60	35	5		100	70	30			100
	ANTI	63	19			82	79	10			89	18	23	50		92	59	28			87
	CBZ	65	21			86	70	20			90	50	35			85	52	33	2		87
acidic	SAL AC			35	25	59			4	17	21			90		90				19	19
	CLO AC			88	19	107			91		91			85		85			85		85
	FEN			91		91			91		91			89		89			91		91
	DICLO			80		80			80		80			83		83			73		73
	IBP			92		92			97		97	1	2	83		86		6	93		99

w1: 1 mL MeOH, w2: 1 mL MeOH, e_a: 5 mL 5% HCOOH in MeOH, e_b: 5 mL 5% NH_4OH in MeOH, Σ: total sum of the recoveries of the four fractions.

step, suggesting that these compounds with basic and acidic properties establish strong ionic interactions with the sorbents in the prepared cartridges. Furthermore, the analytes were rinsed in the acidic or the basic elution fractions exactly as predicted by their pK_a values (Table 2). For instance, in the SAX/SCX configuration, the acidic pharmaceuticals eluted in the acidic elution, while the basic pharmaceuticals eluted in the basic elution. In the case of the SCX/WAX configuration, all of the analytes were rinsed during the basic elution. No differences were observed in the retention of the selected analytes with regard to the strong and weak character of the sorbents included in the cartridges, showing that all of the combinations of the sorbents work well as long as the SPE conditions are properly chosen.

It must be highlighted that, when combining sorbents using the same weight rather than the same ion exchange capacity (meq), the charge in excess had an effect on the analytes with the same charge, showing, for instance, how acids were partially lost in the washing fraction (data not shown) when the negative charge was predominant in the cartridge. In summary, the potential of applying combinations of mixed-mode sorbents to extract basic and acidic analytes simultaneously was demonstrated, using a simple SPE procedure with the advantage of eliminating neutral interferences in the washing step.

Because the SAX/WCX cartridge displayed slightly less reproducibility for the basic pharmaceuticals compared to others, and the WAX/WCX cartridge has a very narrow range of pH at which both the analytes and sorbents are charged, the most promising combinations were the SCX/SAX and SCX/WAX cartridges. These two configurations were used to evaluate if the preliminary conditions predicted as most favorable were in fact optimal, by studying the effect of changing the SPE parameters in the performance of the extraction. In addition, the use of conditions that, in theory, should not work properly will further confirm the presence of ionic interactions as the driving force of the retention of the charged analytes.

3.2.2. Influence of pH on retention

The effect of changing the pH of the loading and elution steps was studied in both the SCX/SAX and

the SCX/WAX cartridges, as these parameters are very important in the overall procedure. Initially, different pH values (2, 7, 10 and 12) were tested in the loading step, following the same SPE procedure described in the previous section, for both the SCX/SAX and SCX/WAX cartridges. In theory, for the SCX/SAX cartridge, the optimal loading pH range is ~5 to 8, because acidic pharmaceuticals are in their neutral form at pH 2 and basic analytes are uncharged at pH 10 and 12 (Figure 1). Table 3 shows how acidic pharmaceuticals were lost in the washing step when loading at pH 2, while basic ones were lost in the washing step when loading at pH 12, just as expected. In this table, only the recoveries obtained for pH 2 and 12 are shown, as these were the pH conditions under which ionic interactions were weakened. It was also expected that, when loading at pH 5 and 7, all pharmaceuticals (with the exception of the neutral ones) were retained through ionic interactions. At pH 10, basic pharmaceuticals were partially lost in the washing step (7% to 23%) rather than being completely lost,

Table 3. Recoveries (%) obtained in ultrapure water when the SCX/SAX and the SCX/WAX configurations were loaded at pH 2 and 12.

		pH2					pH12					
		SCX/SAX			SCX/WAX		SCX/SAX			SCX/WAX		
	Analyte	w	e_a	e_b	w	e_b	w	e_a	e_b	L	w	e_b
basic	ATE			79		85	63		12		54	30
	RAN			84		86	77		2		78	6
	TRI			82		84	82				83	
	MET			82		83	59		15		53	30
	PROP	1		89		90	48	1	36		44	46
neutral	CAFF	56			67		69				84	
	ANTI	15	19	37	3	78	79				87	
	CBZ	86			85		87				89	
acidic	SAL AC	9	28	12		93		16	9	69	1	
	CLO AC	44	39		12	71		86		78	11	
	FEN	81	6		75	16		85			91	
	DICLO	55	25		22	58		83			92	
	IBP	84			84			87			81	

L: loading, w: w1 + w2 (1 mL MeOH + 1 mL MeOH), e_a: 5 mL 5% HCOOH in MeOH, e_b: 5 mL 5% NH_4OH in MeOH.

as expected. This was not surprising, as pH 10 is around the pK_a values of these analytes, so the test was performed at the limit of the conversion between the ionic and the neutral form.

For the SCX/WAX cartridge, the optimal loading pH range is ~5 to 6, because acidic pharmaceuticals are in their neutral form at pH 2 and the piperazine group of the WAX sorbent is in its neutral form from pH ~6 upwards, as stated by the manufacturer of the sorbents (Figure 1). In Table 3, it can be seen that both acidic and basic pharmaceuticals were lost in the washing step when loading the sample at pH 12, while, at pH 2, it was the acidic analytes that were rinsed in this step, just as expected. At pH 7 (data not shown), the acidic pharmaceuticals were strongly retained during the MeOH wash, rather than losses being observed, as expected. Actually, acidic pharmaceuticals start to experience losses from pH 10, suggesting that the piperazine group might have a higher pK_a within the polymeric network. At this pH, basic pharmaceuticals were also ionically retained, which is explained by the fact that these conditions are at the limit of the pK_a values of these analytes, just as observed before for the SCX/SAX cartridge. Low recoveries observed for SAL AC during these tests was explained by excessive retention on the cartridges, which was resolved by raising the % HCOOH in MeOH from 5% to 10% for the elution in further tests.

The effect of changing the pH of the elution step was also evaluated by interchanging the elution steps established in Section 3.2.1. In the case of the SCX/SAX cartridge, the elution was now performed starting with a basic additive instead of an acidic one, followed by the acidic elution: 1) 5 mL of 5% NH_4OH in MeOH, 2) 5 mL of 10% HCOOH in MeOH, while, for the SCX/WAX configuration, the elution started with the acidic rather than the basic one. As expected, for the SCX/SAX cartridge, basic pharmaceuticals now eluted in the first fraction, while acidic ones eluted in the second fraction with the acidic solution. Thus, the presence of ionic interactions between the analytes and the sorbents was further confirmed. It must be highlighted that, when using this elution order, a percentage (6% to 26%) of CLO AC and IBP started eluting in the basic elution. The theory was also confirmed for the SCX/WAX configuration, because all of the charged pharmaceuticals were not

recovered in the first elution fraction as before. Instead, acids were recovered in the first acidic elution, while bases were collected in the basic elution.

In this section, it was confirmed that the strong retention observed for charged pharmaceuticals was due to the establishment of ionic interactions between the analytes and the charged functional groups of the sorbents. These interactions are only possible in the range of pH values at which both the analytes and sorbents are in their charged form. When the system does not meet these conditions, the ionic interactions are weakened and retention is driven only by hydrophobic interactions.

3.2.3. Optimization of other SPE conditions

Using the same cartridges selected previously, SCX/SAX and SCX/WAX, other parameters of the SPE procedure were evaluated, such as volume in the different steps and washing solvent. To optimize the washing volume, 50 mL of ultrapure water adjusted to pH 5 was loaded in both cartridges, which were then washed with subsequent fractions of 1 mL of MeOH up to 15 mL. It was observed that the neutral pharmaceuticals were lost completely in the first 1 mL washing fraction in both types of cartridges, while no losses were recorded for the rest of the analytes in any of the fractions. Only IBP was partially lost (40%) starting from the seventh fraction. After ascertaining that this loss was due to the selection of a pH value too close to the pK_a of IBP (4.85), and confirming that, at pH 7, the compound was not lost, the loading pH was set at this value from this point onwards. Thus, the washing volume was set at 15 mL of MeOH because no losses were observed for any of the target analytes and a volume higher than this was considered excessive. Actually, this volume of 15 mL is already high for the amount of sorbent used (100 mg) and, as such, it was expected to eliminate a large number of interferences in complex matrices.

In subsequent tests, 50 mL of ultrapure water adjusted to pH 7 was loaded in both cartridges, which were further washed with ACN instead of MeOH, to evaluate the influence of a different organic modifier. No differences were observed between the two organic solvents in any of the cartridges. As MeOH is most commonly used for the washing steps in mixed-mode SPE procedures, it was used

for further extractions. The elution volume was optimized by passing 1 mL fractions up to 5 mL of the elution solvents as follows, for SCX/SAX: 1) 10% HCOOH in MeOH solution followed by 2) 5% NH_4OH in MeOH solution; as for SCX/WAX: 5% NH_4OH in MeOH. For both cartridges, the volume initially selected of 5 mL of each eluting solution proved to be enough to completely elute all of the analytes, so it was considered optimal.

For both configurations, SCX/SAX and SCX/WAX, the SPE protocol established to this point was transferred to 500 mg cartridges, which allowed the loading of 100, 250 and 500 mL of ultrapure water (adjusted to pH 7) to then be washed with 15 mL of MeOH. As a result, up to 500 mL could be passed through the cartridges without observing losses of any of the analytes during the loading or washing step, obtaining recoveries that ranged from 84% to 97%.

3.3. Environmental water samples

The optimized SPE procedures for each type of cartridge, SCX/SAX and SCX/WAX, were evaluated in river water and wastewaters to observe the performance of the combinations of the sorbents when dealing with complex matrices. The neutral analytes (CAFF, ANTI and CBZ) will not be discussed below, as it was proven that they are lost during the washing step fraction. A considerable ME is commonly observed when determining pharmaceuticals in environmental waters, which increases when using high volumes of sample [37]. Thus, the first parameter evaluated when testing the optimized method in these types of matrices was the sample volume. The optimal volumes are indicated in Section 2.3, selected according to the breakthrough volume of the analytes and the ME observed.

At this point, both the SCX/SAX and SCX/WAX cartridges were compared with regard to their performance in environmental waters to select a single configuration for subsequent tests. The optimal procedure described in Section 2.3 was applied to analyse 100 mL of effluent wastewater sample using the two types of cartridges. The results are shown in Table 4. The $\%R_{SPE}$ was defined as the recovery obtained only in the SPE procedure and it was calculated as the ratio between the concentrations obtained from

Table 4. Comparison of the performance between the SCX/SAX and SCX/WAX configurations for extracting the selected basic and acidic pharmaceuticals from 100 mL of effluent wastewater.

		SCX/SAX			SCX/WAX		
	Analyte	**%$R_{apparent}$**	**%R_{SPE}**	**%ME**	**%$R_{apparent}$**	**%R_{SPE}**	**%ME**
basic	ATE	53	104	-49	55	105	-48
	RAN	51	60	-15	52	69	-25
	TRI	50	97	-48	54	97	-44
	MET	70	84	-17	73	91	-20
	PROP	68	83	-18	58	79	-27
acidic	SAL AC	45	62	-28	79	109	-27
	CLO AC	88	97	-10	88	105	-17
	FEN	72	95	-24	74	102	-28
	DICLO	74	101	-27	76	104	-27
	IBP	68	96	-28	47	68	-31

a sample spiked before and after the SPE procedure. The ME was calculated from the concentration obtained when the extract of the sample was spiked just before injection into the LC-HRMS. This concentration (C_{exp}) was introduced in the formula %ME = $[(C_{exp}/C_{theo})*100] - 100$, where C_{theo} is the theoretical concentration in the final volume of sample injected in the LC-HRMS instrument. The $\%R_{apparent}$ was defined as the recovery of the whole method and it was calculated from the concentration obtained from a sample spiked at the beginning of the complete analysis. All of the experimental concentrations mentioned were calculated using a calibration curve prepared in pure standard.

It can be observed in Table 4 that, for the SCX/SAX configuration, values of $\%R_{SPE}$ were between 83% and 104%, with the exception of RAN (60%) and SAL AC (62%), similar to the SCX/WAX configuration, which showed values between 79% and 109%, except for RAN (69%) and IBP (68%). $\%R_{apparent}$ values ranged from 45% to 88% and from 47% to 88% for the SCX/SAX and SCX/WAX cartridges, respectively. The ME was below -28% in both cartridges, except for ATE and TRI, which showed values around -48%. Clearly, no differences were observed between both types of configurations, suggesting that either of them can be equally used for simultaneously extracting the

charged pharmaceuticals from the samples. For further tests, the SCX/SAX was selected from the two types of sorbents because it gave a higher $\%R_{apparent}$ for IBP, which is one of the analytes with a lower response in the LC-HRMS.

Using the SCX/SAX configuration, the ME was compared when extracting effluent and influent wastewaters, and the washing step was applied or omitted, to determine the efficiency of including this cleaning step in the SPE procedure. Figure 2 shows the results of this evaluation, demonstrating a decrease in the ME obtained for several of the analytes, especially basic pharmaceuticals, when the washing step was included. The ME obtained when applying the washing step ranged between -49% and -15% for effluent wastewater, and between -51% and -20% in the case of influent wastewater, which was higher than expected considering that the volume used for the washing step (15 mL MeOH) was quite high. These results can be attributed to the presence of a high content of ionic interferences in the samples that contribute to the ME to a high degree.

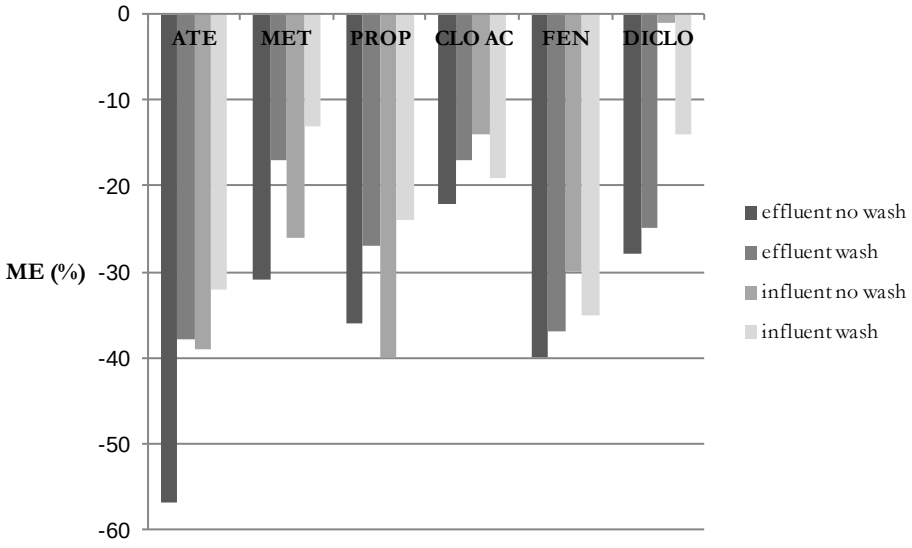

Figure 2. ME (%) obtained for 100 mL of effluent (spiked at 5 mg/L) and 50 mL of influent (10 mg/L) wastewater samples when applying or not the washing step (15 mL of MeOH) of the optimized SPE procedure.

Table 5. $\%R_{apparent}$, $\%R_{SPE}$ and ME values obtained for 100 mL of river water and 50 mL of influent wastewater sample as well as %RSD (n = 5) and MOLs values.

Analyte	River					Influent				
	$\%R_{apparent}$	$\%R_{SPE}$	%ME	%RSD	MQL (ng/L)	$\%R_{apparent}$	$\%R_{SPE}$	%ME	%RSD	MQL (ng/L)
ATE	109	104	-5	3	1	61	102	-42	1	15
RAN	68	83	-25	11	20	71	79	10	1	110
TRI	98	94	-4	5	2	67	98	-35	1	120
MET	102	93	5	2	2	70	93	-24	8	115
PROP	90	90	-3	16	2	70	97	-33	10	115
SAL AC	50	92	-34	4	20	34	57	-40	14	120
CLO AC	65	90	-21	16	2	76	101	-19	22	35
FEN	45	76	-36	6	5	49	99	-41	8	15
DICLO	48	74	-32	18	2	62	107	-24	4	15
IBP	31	60	-41	14	25	38	116	-61	2	265

The SPE procedure was further evaluated in river and wastewater samples in terms of $\%R_{SPE}$ and $\%R_{apparent}$. The results obtained for river and influent are summarized in Table 5. Great $\%R_{SPE}$ values were obtained for all the matrices, being higher than 90% in 70% of the cases. Values of $\%R_{apparent}$ for river water samples were between 45% and 109%, with the exception of IBP (31%), while, for effluent and influent wastewater samples, values ranged from 52% to 83% (except for SAL AC, which were 30%) and from 34% to 76%, respectively. The ME obtained with the present method can be attributed only to ionic interferences. Roughly, the results obtained when using combined sorbents were similar to or better than other reported studies [9, 24, 38]. More importantly, the method demonstrated the capability of simultaneously retaining acidic and basic analytes and the advantage of eliminating all neutral interferences, features that could be transferred to other groups of ionizable compounds and highly complex matrices.

3.4. Method validation and application to environmental samples

The optimized method using the SCX/SAX cartridge was validated in river water and effluent and influent wastewater samples to check its repeatability and detection and quantification limits. Repeatability was expressed as the relative standard deviation (%RSD, n=5) and it was evaluated within the same day (results shown in Table 5) and on consecutive days (<25% in all cases). The method exhibited satisfactory precision, as %RSD values ranged between 0.4% to 24% for all of the pharmaceuticals in all matrices.

Because several of the target analytes were present in the blanks of wastewater samples, the use of matrix-matched calibration curves to correct the ME was not possible for these matrices. In these cases, external calibration curves were used for quantification, considering $\%R_{apparent}$ values into consideration. In the case of river water, a matrix-matched calibration curve was prepared by spiking at different concentrations 100 mL volumes of river water, which were extracted in the SCX/SAX cartridges and subsequently injected into the LC-HRMS instrument. Linearity was great for all of the compounds (R^2 ≥0.9988) between the MQLs (reported in Table 5) and 500 ng/L, except for RAN, SAL AC and IBP, which showed poor

linearity in the concentration range tested. In these cases, external calibration curves considering $\%R_{apparent}$ values were used for quantification, as proposed for wastewater samples.

For wastewater samples method, detection (MDLs) and quantification (MQLs) limits were estimated from the instrumental limits (LODs and LOQs), taking into account the $\%R_{apparent}$ values. For river water samples, MDLs were the spiked concentrations that showed a signal for the more abundant fragment around $1x10^3$, while MQLs were the first points of the matrix-matched calibration curves. MDLs were between 0.5 and 5 ng/L for river water samples, while, for effluent and influent wastewater samples, values were between 1 and 75 ng/L and between 3 and 260 ng/L, respectively. Table 5 shows the MQLs values, which ranged from 1 and 25 ng/L for river water samples, while, for effluent and influent wastewater samples, values ranged from 5 to 80 ng/L and from 15 to 265 ng/L, respectively. The average values reported in the literature for MDLs and MQLs are around the values obtained in the present study [10, 30, 38].

Three different samples of effluent and influent wastewater and two different samples of river water were analysed using the validated method for the SCX/SAX combination. The ranges of concentrations found are shown in Table S2. In river samples, several compounds were detected, but only ATE, MET, SAL AC and CLO AC were quantified at concentrations between 1 and 50 ng/L. Most of the compounds were quantified in effluent and influent wastewater samples with levels ranging from 20 to 2,500 ng/L in the case of effluent wastewater samples and from 40 to 50,000 ng/L in the case of influent wastewater. The pharmaceuticals found at the highest concentrations were ATE, SAL AC, DICLO and IBP and levels found for all of the analytes were in line with those reported in the literature [4, 30, 39].

4 Conclusions

The four combinations tested of SCX, WCX, SAX, and WAX sorbents, by pairs with opposite charge, simultaneously and strongly retained basic and acidic pharmaceuticals, as long as the charges are balanced and the SPE conditions are carefully selected. No substantial differences were observed between the four combinations evaluated,

suggesting that whether the combinations include functional groups with strong or weak properties, they have the potential to retain the selected analytes ionically. The correct selection of the pH value used to load the samples and elute the analytes was very important in terms of favoring the ionic interactions between the analytes and the sorbents. In the evaluation of the influence of pH, the retention behavior of the analytes was as expected, confirming that the strong retention by ionic interactions was only possible when both the sorbents and the analytes were charged, according to their corresponding pK_a values.

The strong retention of the analytes on the sorbents allowed the introduction of a washing step with a high volume of MeOH (15 mL), which proved to eliminate neutral interferences present in the matrices. As indicated by the results obtained when extracting environmental waters using the present method, ionic interferences contribute to the matrix effect to a high degree. The performance of the method was comparable to other studies reported and it was validated in river and wastewater samples. Several of the selected analytes were successfully quantified in the samples in levels that were similar to those reported in other studies.

The potential of combining sorbents to obtain positive and negative charges in the same SPE cartridge was confirmed and optimal extraction conditions were given to obtain the best performance. Promising results might be expected for other basic or acidic compounds and other samples with complex matrices.

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Supplementary Data

Table S1. Chemical structure, pK_a values and exact masses of the studied analytes.

Analyte	Formula (M)	Structure	pKa[a]	Molecular ion (m/z)	Fragment Formula (exact mass, m/z)
Atenolol (ATE)	$C_{14}H_{22}N_2O_3$		9.67	$[M+H]^+$ 267.17032	$C_{10}H_9O^+$ (145.06479)
Ranitidine (RAN)	$C_{13}H_{22}N_4O_3S$		8.08	$[M+H]^+$ 315.14854	$C_5H_{10}N_3O_2S^+$ (176.04882)
Trimetoprim (TRI)	$C_{14}H_{18}N_4O_3$		10.8	$[M+H]^+$ 291.14517	$C_{12}H_{13}N_4O_3^+$ (261.09822)
Metaprolol (MET)	$C_{15}H_{25}NO_3$		9.67	$[M+H]^+$ 268.19072	$C_6H_{14}NO^+$ (116.10699)
Propanolol (PROP)	$C_{16}H_{21}NO_2$		9.7	$[M+H]^+$ 260.16451	$C_6H_{14}NO^+$ (116.10699)
Caffeine (CAFF)	$C_8H_{10}N_4O_2$		-1.2	$[M+H]^+$ 195.08765	$C_6H_8N_3O^+$ (138.06619)

Table S1. (Cont.).

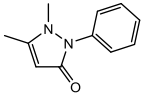
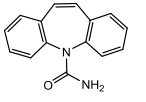
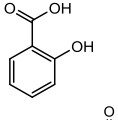
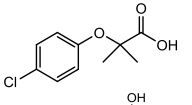
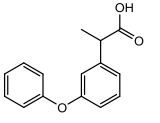
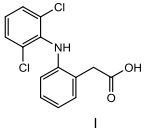
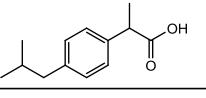
Analyte	Formula (M)	Structure	pKa[a]	Molecular ion (m/z)	Fragment Formula (exact mass, m/z)
Antipyrine (ANTI)	$C_{11}H_{12}N_2O$		0.5	$[M+H]^+$ 189.10224	$C_7H_6N^+$ (104.04948)
Carbamazepine (CBZ)	$C_{15}H_{12}N_2O$		16.0	$[M+H]^+$ 237.10224	$C_{14}H_{12}N^+$ (194.09643)
Salicilic acid (SAL AC)	$C_7H_60_3$		2.79	$[M-H]^-$ 137.02442	$C_6H_5O^-$ (93.03459)
Clofibric acid (CLO AC)	$C_{10}H_{11}ClO_3$		3.37	$[M-H]^-$ 213.0324	$C_6H_4ClO^-$ (126.99562)
Fenoprofen (FEN)	$C_{15}H_{14}O_3$		3.96	$[M-H]^-$ 241.08702	$C_6H_5O^-$ (93.03459)
Diclofenac (DICLO)	$C_{14}H_{11}NCl_2O_2$		4.00	$[M-H]^-$ 294.00941	$C_{13}H_{10}NCl_2^-$ (250.01958)
Ibuprofen (IBP)	$C_{13}H_{18}O_2$		4.85	$[M-H]^-$ 205.1234	$C_{12}H_{15}^-$ (159.11792)

Table S2. Concentration ranges found in river water and wastewater samples when using the validated method with the SCX/SAX combination of sorbents.

Analyte	River (ng/L)	Effluent (ng/L)	Influent (ng/L)
ATE	< MQL	1037 - 2551	2249 - 2927
RAN	< MQL	< MQL	< MQL
TRI	< MQL	237 - 469	178 - 325
MET	3 - 4	80 - 208	135 - 407
PROP	< MQL	124 - 134	142 - 150
SAL AC	52 - 55	MQL -189	398 - 51185
CLO AC	< MQL	21 - 23	39 - 41
FEN	< MQL	< MQL	< MQL
DICLO	< MQL	433 - 905	405 - 1125
IBP	89 - 93	82 - 162	545 - 33406

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3.2.3. Discussion of results

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In this section, the potential of commercial mixed-mode ion-exchange polymeric sorbents was demonstrated to enhance the retention of polar and ionic compounds and to increase their selectivity. Particularly, the results of this Thesis broadened the use of these materials introducing unusual applications, as they proved to retain through ionic interactions a group of neutral compounds or they were combined in single cartridges for the selective and simultaneous extraction of basic and acidic compounds.

Several authors have applied mixed-mode sorbents using protocols that did not exploit the improved selectivity that these sorbents are capable of providing, as no washing procedures with pure organic solvents were included [1, 2]. In other studies, mixed-mode sorbents have been used in their full capability as selective materials, either because the authors follow the recommended protocol or they thoroughly understood the properties of the ionizable analytes and optimized the SPE protocol according to their properties and the sorbents [3, 4]. Nevertheless, there are applications left to explore like the extraction of neutral compounds with the potential capability of developing induced ion-dipoles or the simultaneous extraction of compounds positively and negatively charged.

With this ideas in mind, the first study presented in this section applied mixed-mode sorbents for the retention of a group of analytes that were not ionized at SPE conditions, this is, derivatives of benzotriazole, benzothiazole and benzenesulfonamide. To the extent of our knowledge, this is the first study that thoroughly discusses the possible interactions responsible for the selective retention of neutral compounds on these types of sorbents. Initially, ionic retention was expected only for the pH values at which some of the analytes were able to ionize, but results proved that selective retention was possible at a broad range of pH, and for all the compounds capable of delocalizing electron density, which is the case of benzotriazoles and benzothiazoles. As it was extensively discussed, this electron delocalization probably promoted ionic interactions with the sorbents by creating induced charges within the molecules (Figure 1). This idea was in agreement with the fact that sorbents with both cation and anion-exchange properties were equally successful in selectively retaining the analytes, suggesting that both

negatively and positively charged groups were capable of inducing polarization of the compounds. Furthermore, initial tests on sorbents with weak character showed that they were not able to ionically retain the compounds, indicating the important role that played the nature of the functional groups on the retention. Moreover, the retention behavior observed for the benzenesulfonamides served as an excellent comparison point because they have similar structures but less capability of delocalizing electron density, which lead to a retention based only on reversed-phase interactions.

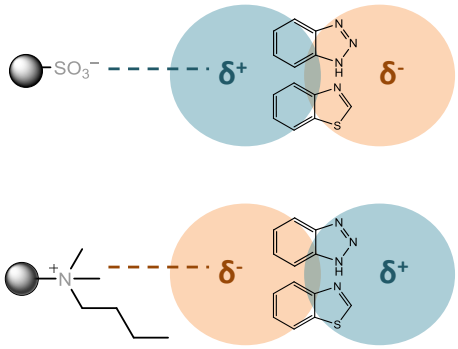

Figure 1. Scheme of possible dipole induced ionic interactions responsible of selective retention of benzotriazoles and benzothiazoles on mixed-mode sorbents with strong cation and anion-exchange character.

From the above observations, other compounds with comparable structure have shown similar results when retained on mixed-mode sorbents. For example, caffeine has been selectively retained on mixed-mode sorbents with cation-exchange properties in spite of being a neutral compound [5, 6]. Probably, the resonance structures of this compound can also contribute to its polarization and therefore its ionic retention. However, in the observations reported in this Thesis caffeine behaved as neutral being always rinsed during washing steps with MeOH. Differences in the type of sorbents and SPE conditions could have been responsible for this behavior,

illustrating the importance of studying the potential of every particular sorbent for the analytes of interest.

Certainly, the application of mixed-mode sorbents as selective materials for the selected compounds in neutral conditions was demonstrated, which could open possibilities for analytes with similar structures.

As previously discussed, mixed-mode sorbents were also used to simultaneously and selectively retain bases and acids by combining an equimolar amount of cation and anion-exchangers in the same cartridge. This configuration helped to apply the simple protocol (loading at suitable pH, washing with methanol and elution at a suitable pH) normally used in single commercial exchangers, retaining together basic and acidic compounds through ionic interactions, while allowing the washing of interferences retained by hydrophobic interactions.

The most important result of this study was the agreement of the observations with the pK_a values of the analytes and the functional groups of the sorbents, confirming the predicted SPE conditions hypothetically proposed as optimum. This observation is in contrast with the results obtained for the benzotriazoles which, being charged at basic pH values (pK_a ~8), should undergo enhanced retention when increasing pH. Instead, their retention was not substantially affected by pH changes. Clearly, the properties of the analytes play an important role during retention beyond their pK_a values, for which the observations made during optimization steps are very important when evaluating these types of sorbents.

Another important observation was the similarity of the results obtained for the four configurations, even when differences were expected between them because of the different chemistry that the functional groups with strong and weak properties have. Instead, the only difference between configurations was the working pH ranges where both the analytes and the functional groups of the sorbents were in their charged form, being narrower for configurations containing groups with weak properties. However, this fact

does not represent any problem if the sample is simply adjusted to the most suitable pH.

Another important result was the influence of combining the sorbents to obtain an equimolar ratio in comparison to mixing the same weight of polymer. In Table 1, the recoveries obtained when loading 50 mL of a mixture of the analytes in ultrapure water adjusted at pH 5 are detailed for two different cartridges combining SCX/SAX sorbents in equimolar or 50/50 (w/w) ratio. When combining both sorbents in an equimolar ratio, both the positive and negative charges have around 0.0220 meq, while by mixing 50 mg of both SCX and SAX sorbents the negative charges present are four times more than the positive ones (0.05 meq *vs.* 0.0125 meq).

Table 1. Recoveries (%) obtained for the SCX/SAX configuration in both equimolar or 50/50 (w/w) cartridges.

		equimolar SCX/SAX 0.02 meq/0.02 meq				**same weight SCX/SAX 50 mg/50mg**			
	Analyte	**w**	**e_1**	**e_2**	**Σ**	**w**	**e_1**	**e_2**	**Σ**
Basic	ATE			80	80			79	79
	RAN			63	63			74	74
	TRI			77	77			82	82
	MET			83	83			83	83
	PROP			80	80			86	86
Acidic	SAL AC		45	19	64		39		39
	CLO AC		86		86		51	8	59
	FEN		87		87	47	2		49
	DICLO		82		82		45		45
	IBP		88		88	77			77

w: 15 mL MeOH, e_1: 5 mL 5% HCOOH in MeOH, e_2: 5 mL 5% NH_4OH in MeOH, Σ: total recoveries in all fractions

When using the cartridge with the same weight of sorbents, recoveries were lower than the ones obtained for the equimolar cartridge, and FEN and IBP were rinsed during the washing step. This data suggested that the retention of

acidic compounds was affected by the excess of negative charges in the sorbent in comparison to positive charges. Further tests at different pH values or using different analytes could give more insights into how this influence of the charge in excess can substantially affect retention, but from this comparison the equimolar cartridges were preferred. Certainly, when using this cartridge configuration the analytes are strongly retained even when 15 mL of MeOH is passed to wash the sorbent; a volume that is quite high for the amount of sorbent used.

As already stated, the advantage of including a washing step with pure organic solvent relies in the possibility of eliminating compounds retained through hydrophobic interactions from the matrix, which in most of the cases lead to a reduction in matrix effect. The values of matrix effect obtained in the first study (~20 %) were globally better than those obtained for the pharmaceuticals in the second study (~30 %). These observations can be attributed to the nature of the analytes or the fact that in the second study both cationic and anionic interferences are also retained together with the target analytes. In opposition, in the method for benzotriazoles and benzothiazoles, interferences with the same charge as the sorbent are eliminated during the washing step. For this reason, matrices rich in ionic interferences could exhibit high matrix effect in combined ion-exchange cartridges, a limitation that could be addressed by testing a particular sorbent configuration or loading pH at which ionic interferences are no longer ionically retained. Moreover, they could be eliminated during aqueous washing steps at a suitable pH. Indeed, every particular system must be carefully evaluated because several applications could benefit from the elimination of neutral interferences in terms of matrix effect.

To the best of our knowledge, this is the first study combining positive and negative charges within the same SPE cartridge, opening possibilities that can be explored for several compounds and matrices. Future research can be focused on this sense, the improvement of SPE protocols to maximize selectivity and elimination of interferences or the development of new sorbents with enhanced technologies.

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3.3. Development of mixed-mode zwitterionic-exchange polymeric sorbents

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
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In the previous section it was demonstrated that having both positively and negatively charged functional groups in the same solid-phase extraction (SPE) cartridge was successful in simultaneously and selectively retain acidic and basic compounds. In addition, the best SPE conditions were settled for the selective retention of ionizable compounds and the elimination of neutral ones and it was observed that the combination of sorbents with either strong or weak properties were equally suitable.

Given the good results obtained in the previous studies and the information gathered concerning the retention behavior, the development of polymeric mixed-mode sorbents with zwitterionic moieties can be another strategy to promote simultaneous ionic retention of basic and acidic compounds. To the best of our knowledge, none polymeric material with these properties have been applied for SPE purposes. Polymeric materials functionalized with zwitterionic moieties have been recently applied as HILIC stationary phases (Section 1.1.2.1). In the present section, the synthesis of polymeric microspheres functionalized with zwitterionic groups for SPE is described. Different materials with interesting properties could be obtained if a single ligand containing positive and negative charges is introduced in the polymeric particles rather than merely blending cation and anion-exchangers. The polymer could benefit from interactions between the charged groups of the zwitterionic moiety or the configuration of the polymeric network and accessibility to the ionic functionalities can be different from the combined particles.

The development of these novel materials was possible thanks to experience gained from the collaborative work that our research group has done with the Polymer Group led by Professor Peter A. G. Cormack of the University of Strathclyde for more than 15 years, on the synthesis of new functional materials [1]. Continuing with this fruitful collaboration, the sorbents were prepared during the research stay in the laboratory of the Polymer Group. In particular, the starting point for the synthesis described in this section was the promising results obtained in the work developed by Anderson *et al.* [2] in Cormack's group.

Among the polymerization methods used to produce polymeric microspheres, precipitation polymerization has proved to yield highly crosslinked macroreticular particles with high specific surface area (up to 800 m^2/g), narrow particle size distribution and low particle diameter (2–5 μm) which are desired features in SPE sorbents to improve capacity. Because this synthetic approach requires a homogeneous mixture of monomer, solvent and initiator without the use of stabilizers or surfactants, the resulting product is cleaner than other types of polymerizations [3]. As previously exposed in the Introduction, polymeric microspheres synthesized from divinylbenzene (DVB) and vinylbenzyl chloride (VBC) have pendent chloromethyl groups that can react in hypercrosslinking (HXL) reactions which highly increase the surface area (up to 1200 m^2/g) of the polymeric network, by forming new methylene bridges through the Friedel–Craft reaction and the aromatic benzene rings [1].

There are several strategies to functionalize polymeric microspheres with ionic groups which are generally amine or acidic groups with strong or weak properties. One approach is the introduction of a comonomer with the desired functionality in the reaction mixture for the polimerization, which should yield the polymer particles with the functional groups already incorporated [4-6]. This is the fastest methodology as the functionalized polymer is obtained in one single step. However, this strategy would require thorough optimization as the introduction of an ionic comonomer to the polimerization mixture could prevent the incorporation of a desired monomer, introduce solubility problems or affect precipitation of the particles [7].

Another strategy is the introduction of functional groups after the polymerization reaction. In materials based on DVB, a wide variety of post-polymerization modifications can be performed thanks to the reactivity and accessibility of aromatic rings. The aromatic π-system is rich in electron density being susceptible to electrophiles in electrophilic aromatic substitution reactions [8, 9]. Strong cation-exchangers have been developed using this approach with reagents like sulfuric acid (H_2SO_4), acetyl sulfate or lauroyl sulfate [6, 9].

Pending chloromethyl groups of both macroreticular and HXL particles can also be exploited for the functionalization of the polymer beads in substitution reactions with a suitable nucleophile. Several anion-exchangers have been synthesized using this strategy, promoting the reaction of the microspheres with a desired amine under basic conditions [10, 11]. The latter approach was used for the synthesis of three new materials with zwitterionic moieties, described in the following section. For two of these materials, reagents containing amine groups were used as nucleophiles to substitute the pending chloride groups of the polymer particles. After the introduction of the amino functionality, the resulting polymers were subjected to a second reaction to introduce the acidic functionality (in the same ligand) and obtain the amino acid properties. In the case of the third material, the zwitterionic moiety was introduced at once as the reagent contained together amino and acidic groups.

The developed materials with zwitterionic properties were evaluated from the analytical perspective for the extraction of a group of pharmaceuticals, drugs of abuse and artificial sweeteners, selected as model compounds for their chemical structures as well as their basic, neutral or acidic properties. Special attention was paid to the retention behavior of the different compounds at different SPE conditions such as pH and types of solvents to understand the interactions promoted by the synthesized zwitterionic sorbents.

The paper discussing the results of the present study is going to be submitted for publication in the *Journal of Chromatography A*.

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3.3.1. Synthesis and evaluation of polymeric sorbents with zwitterionic character for the solid-phase extraction of model compounds with acidic and basic properties

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STRATEGIES TO IMPROVE THE DETERMINATION OF POLAR COMPOUNDS IN ENVIRONMENTAL WATERS
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SYNTHESIS AND EVALUATION OF POLYMERIC SORBENTS WITH ZWITTERIONIC CHARACTER FOR THE SOLID-PHASE EXTRACTION OF MODEL COMPOUNDS WITH ACIDIC AND BASIC PROPERTIES

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Abstract

Mixed-mode ion-exchange polymers as sorbents for solid-phase extraction (SPE) have attracted attention thanks to their ability to retain compounds through a combination of hydrophobic and ionic interactions. To date, both cation- and anion-exchangers have been exploited successfully as extraction sorbents, however the application of SPE sorbents bearing both cation- and anion-exchange moieties simultaneously within the same sorbent (zwitterionic polymers) has been little explored. In this study, the synthesis of three mixed-mode zwitterionic sorbents based on microporous polymer microspheres of poly(divinylbenzene-*co*-vinylbenzyl chloride) polymer backbones is disclosed. The synthesized sorbents were evaluated in terms of their suitability for SPE. Three groups of compounds with different structures and acidic or basic properties were selected to study their retention behavior on the sorbents, to gain insights into the properties of the materials and the optimum extraction conditions for each analyte. The most promising sorbent had weak cation- and anion-exchange character, and retained certain strongly acidic and basic compounds. Thus, the present study introduces the concept and application of polymers with zwitterionic character as SPE sorbents.

Keywords: *zwitterionic polymers; polymer beads; hypercrosslinked; solid-phase extraction; acidic and basic analytes; selectivity.*

1 Introduction

The development and application of new sorbents for solid-phase extraction (SPE) is an ongoing and growing research area because SPE is established as the sample treatment technique of choice for liquid samples in several analytical methods [1-4]. The advantages of SPE over other techniques include the possibility of enriching the sample, achieving high recoveries, and the ability to select the most appropriate sorbent for any specific application [5, 6]. From an analytical perspective, an ideal sorbent is one that must give complete recoveries of analytes and be able to selectively retain the target analytes during clean-up steps whilst excluding interferences. The removal of interfering compounds is particularly important when using methods that include liquid chromatography (LC) coupled to mass spectrometry (MS) based detectors, because the MS response is usually affected by the matrix effect [7-9]. In several cases, the samples of interest have quite complex matrices and the target analytes are found at trace levels, as is the case for many environmental and biological samples [10-12]. For this reason, the design of new sorbents for several applications has focused on improving both their capacity and selectivity, by increasing their specific surface area and incorporating functional groups that can promote selective interactions with the analytes [13].

The use of mixed-mode ion-exchange polymeric sorbents for treating complex samples is very attractive, as has been demonstrated in several studies [8, 9, 11, 13-18]. These sorbents combine a porous polymeric network with ionic functional groups promoting reversed-phase and ionic interactions within the same material [13, 19-21]. Commercial suppliers, such as Waters and Phenomenex, offer several mixed-mode sorbents, including cation- and anion-exchangers with strong or weak interactions (SCX or WCX and SAX or WAX). These sorbents are typically based upon macroreticular poly(divinylbenzene-*co*-vinylpyrrolidone) or poly (styrene-*co*-divinylbenzene) with particle diameters between 30 and 100 μm [22]. Furthermore, the application of novel mixed-mode sorbents prepared in academic laboratories has also been reported [13]. Within our research group, several in-house mixed-mode sorbents have been demonstrated to enrich the analytes and eliminate interferences from environmental water samples,

achieving high recoveries and reducing the matrix effect in LC-tandem mass spectrometry (MS/MS) instruments [23, 24]. These sorbents are based typically on hypercrosslinked or macroreticular polymer microspheres with low particle diameters (a few microns), narrow particle size distributions and very high specific surface areas, features that led to performance outputs superior to the commercially available sorbents [13]. However, both commercial and in-house prepared materials are currently limited to the selective extraction of either cationic or anionic analytes, provided that a clean-up step is included which removes neutral compounds as well as compounds with the same charge as the sorbent.

Some authors have attempted to combine mixed-mode sorbents to simultaneously extract cationic and anionic compounds. For this purpose, they designed protocols whereby cartridges containing the sorbents were placed in tandem; satisfactory results were reported, although the extraction procedure was slow (a doubling of the time) [25, 26]. In an earlier paper [27], successful results were obtained when physically combining mixed-mode sorbents to obtain single cartridges with functional groups positively and negatively charged, with the objective being to develop a simple SPE protocol to extract basic and acidic compounds simultaneously. With this in mind, the aim of the present study was to use zwitterionic polymers, prepared in-house, for the simultaneous extraction of basic and acidic compounds, an approach which would broaden the potential of mixed-mode sorbents and potentially simplify SPE protocols. The synthesis and characterization of three distinct sorbents containing zwitterionic moieties is described. Three groups of basic, neutral and acidic compounds with different structural properties were then selected and used as model compounds to be extracted on the sorbents to evaluate the sorbents from an analytical perspective.

2 Experimental

2.1. Reagents and standards

For the synthesis of the polymer microspheres, the monomers used were divinylbenzene (DVB) (80 % technical grade) and 4-vinylbenzyl chloride (VBC) (90 % technical grade), both supplied by Sigma-Aldrich (England, UK) and passed through a short column of

alumina (activated, neutral, also supplied by Sigma-Aldrich) prior to use. 2,2´-Azobis(isobutyronitrile) (AIBN) (97%), supplied by BDH Lab Supplies (Poole, UK), was recrystallized from cold acetone at low temperature. Iron (III) chloride (96% anhydrous) was also obtained from BDH Lab Supplies and was used for the hypercrosslinking reactions. Acetonitrile (ACN) (99.9% HPLC grade), toluene (99.3% Lab Reagent), 1,2-dichloroethane (DCE) (99.8% anhydrous), acetone, dichloromethane, methanol (MeOH) (≥ 99% Analytical specification), diethyl ether (99.8% ACS reagent), ethanol (EtOH) (≥ 99.8%) and tetrahydrofuran (THF) were all purchased from Sigma-Aldrich. Nitric acid (65%, supplied by Sigma-Aldrich), potassium carbonate (K_2CO_3), sodium hydrogen carbonate ($NaHCO_3$) and sodium chloride (NaCl), supplied by VWR International (Leuven, Belgium), were used to prepare the washing solutions for cleaning the products. For the polymer analogous reactions, the reagents used were sarcosine ethyl ester hydrochloride, taurine, imidazole, 1,3-propanesultone and triethylamine (TEA), all sourced from Sigma-Aldrich and of high purity (≥99%), tetrabutyl ammonium hydroxide solution (~40% in H_2O) also supplied by Sigma-Aldrich, and potassium hydroxide (KOH) obtained from VWR International.

The group of model compounds selected for the SPE evaluation of the sorbents were 13 pharmaceuticals: atenolol (ATE), ranitidine (RAN), trimethoprim (TRI), metoprolol (MET), propranolol (PROP), caffeine (CAFF), antipyrine (ANT), carbamazepine (CBZ), salicylic acid (SAL AC), clofibric acid (CLO AC), fenoprofen (FEN), diclofenac (DICLO) and ibuprofen (IBP); 7 drugs of abuse: morphine (MOR), codeine (COD), mephedrone (MMC), benzoylecgonine (BE), methylene dioxypyrvalerone (MDPV), cocaine (COC) and methadone (MET); and 7 artificial sweeteners: alitame (ALI), neotame (NEO), potassium acesulfame (ACE), saccharin (SAC), aspartame (ASP), neohesperidine dihydrochalcone (NHDC) and glycyrrhizic acid (GLY) ammonium salt; all purchased as pure standards from Sigma-Aldrich (St. Louis, MO, USA). These analytes were selected according to their pK_a values and structures, as detailed in Tables S1, S2 and S3, and by their environmental relevance as emerging organic contaminants. Standard stock solutions were prepared in MeOH for each

analyte at a concentration of 1 mg/mL and were kept at -20 °C. For each group of compounds, one solution with the mixture of the analytes was prepared weekly and stored at 4 °C, at concentrations of 70 mg/L for the pharmaceuticals and 100 mg/L for the drugs of abuse and artificial sweeteners. By diluting the mixed solutions, working solutions were prepared daily in ultrapure water, which was obtained using a water purification system (Veolia, Sant Cugat del Vallès, Spain). Ultra gradient HPLC grade MeOH and ACN were purchased from J. T. Baker (Deventer, the Netherlands). Formic acid (HCOOH) from Sigma-Aldrich, hydrochloric acid (HCl) from Scharlab (Barcelona, Spain) and ammonium hydroxide (NH_4OH) from Panreac (Barcelona, Spain) were used to prepare the mobile phases and solutions for SPE.

2.2. Synthesis of the sorbents

2.2.1. HXLPP-WCX/WAX

The synthesis of this sorbent involved an initial polymerization to yield gel-type polymer microspheres followed by a hypercosslinking reaction to yield polymer microspheres with high specific surface area. The functionalization of the hypercrosslinked polymer microspheres was performed using sarcosine ethyl ester hydrochloride, to introduce the anion-exchange properties to the particles. Subsequently, the polymer was subjected to the hydrolysis of the ester groups to obtain the amphoteric form. The overall synthetic procedure is shown in Figure 1. The preparation of the gel-type poly(DVB-*co*-VBC) microspheres was performed using an adapted precipitation polymerisation (PP) protocol developed in our laboratories [28], by placing both monomers, DVB (2.735 mL, 2.5 g, 19.2 mmol) and VBC (6.925 mL, 7.5 g, 49.1 mmol), together with AIBN as initiator (0.2794 g, 1.7 mmol, 2 mol% relative to the polymerizable double bonds), in a Nalgene® bottle (1 L) containing 500 mL of ACN. The solution was placed in an ultrasonic bath for 15 min and placed subsequently in an ice bath to be deoxygenated by sparging with N_2 for a further 15 min. After deoxygenating, the bottle was sealed immediately and placed on a low-profile roller in an incubator with controllable temperature. The temperature was increased from ambient to 60 °C over 2 h and it was held at this temperature for 48 h. The product was a milky suspension of white

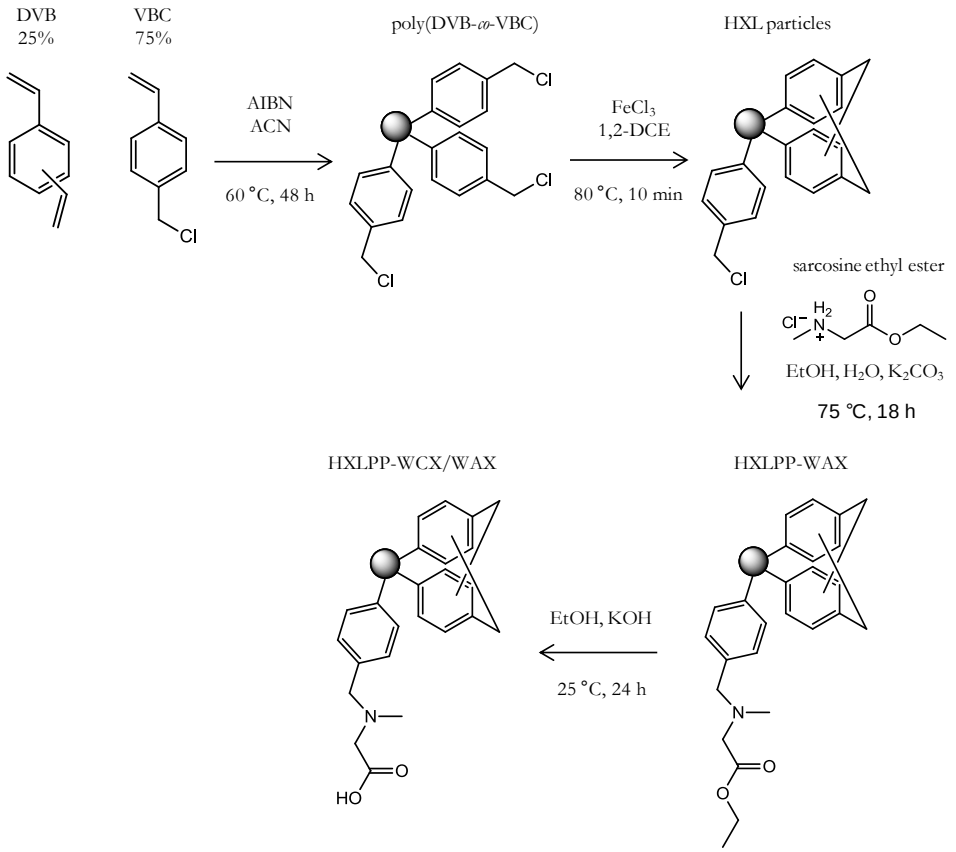

Figure 1. Schematic representation of the synthetic procedure used for the preparation of the HXLPP-WCX/WAX sorbent.

particles. The particles were filtered from the reaction mixture on a 0.2 μm nylon membrane filter and then washed successively with ACN, MeOH, toluene and acetone before being dried overnight in a vacuum oven at 70 °C and 60 mbar (2.4832 g). The resulting polymer microspheres were subjected to a hypercrosslinking procedure derived from previous protocols [28]. The gel-type poly(DVB-*co*-VBC) particles (2.0349 g, 7.3 mmol of Cl) were placed in a round-bottomed flask equipped with a reflux condenser, an overhead stirrer and an oil bath with controlled temperature. DCE (40 mL) was added and the particles were stirred (100 rpm) and left to swell in the solvent

under N_2 at room temperature for 1 h. Iron (III) chloride (1.3049 g, 8.0 mmol) and a second volume of DCE (30 mL) were added to the mixture before heating to 80 °C for 10 min. The particles suspended in the dark purple solution were filtered on a 0.2 µm nylon membrane filter and washed successively with MeOH, 2 M HNO_3 aqueous solution, MeOH and acetone. The orange-coloured particles were washed overnight with acetone in a Soxhlet extractor. Then, the particles were filtered again (0.2 µm nylon membrane filter) and washed with MeOH and diethyl ether and dried overnight in a vacuum oven (70 °C, 60 mbar) (1.7694 g).

For the chemical functionalization, the HXL particles (1.5256 g, 2.2 mmol Cl/g) were placed in a round-bottomed flask equipped with a reflux condenser, an overhead stirrer and an oil bath with controlled temperature. EtOH (40 mL) was added and the mixture was stirred (100 rpm) for 1.5 h. Sarcosine ethyl ester hydrochloride (2.3004 g, 14.97 mmol) and K_2CO_3 (2.0383 g, 14.75 mmol) were dissolved in H_2O (40 mL) and added to the mixture before heating to 75 °C for 18 h. The orange-coloured particles were filtered (0.2 µm nylon membrane filter) and washed successively with EtOH, MeOH, 1:1 MeOH:H_2O solution, 0.01 M $NaHCO_3$ aqueous solution, H_2O and acetone. Then, the particles were dried overnight in a vacuum oven (70 °C, 60 mbar) (1.5610 g).

Finally, the modified particles HXLPP-WAX were modified further by base-catalyzed ester hydrolysis to reveal the carboxylic acid groups. For this purpose, the modified polymer (0.6252 g) was placed in a glass Kimax tube together with an ethanolic solution of KOH, prepared by dissolving 1.0607 g of KOH in 20 mL of EtOH. The tube was sealed and agitated gently on a low-profile roller at room temperature for 24 h. The product HPLXPP-WCX/WAX was filtered (0.2 µm nylon membrane filter) and washed with large volumes of EtOH and then dried overnight in a vacuum oven (70 °C, 60 mbar) (0.6391 g).

2.2.2. PP-SCX/WAX

For the synthesis of this sorbent, macroreticular polymer micro-spheres prepared by PP were used as starting material for the substitution reaction carried out with the tetrabutylammonium salt of taurine (Figure 2).

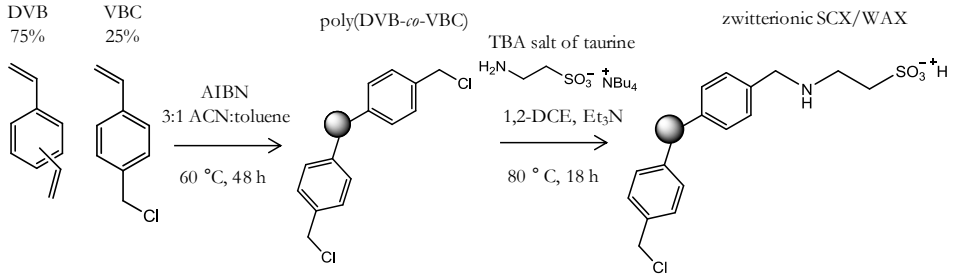

Figure 2. Schematic representation of the synthetic procedure used for the preparation of the PP-SCX/WAX sorbent.

For the synthesis of the macroreticular particles, the comonomers DVB (8.206 mL, 7.5 g, 57.6 mmol) and VBC (2.308 mL, 2.5 g, 16.4 mmol), and AIBN (0.3976 g, 2.42 mmol, 2 mol % relative to the polymerisable double bonds), were added to 500 mL of a 3:1 (v/v) ACN:toluene mixture in a Nalgene bottle (1L). The rest of the procedure was performed as per Section 2.2.1. The tetra-butylammonium salt of taurine was prepared by dissolving taurine (1.5065 g, 11.9 mmol) in a minimum volume of water and adding 6 mL of technical grade tetrabutylammonium hydroxide solution (~40% in H_2O) to the taurine solution. The solution was stirred for 1 h at room temperature and evaporated subsequently under reduced pressure at 70 °C. The wet solid obtained was placed in a heated dessicator under vacuum overnight. The dried solid was dissolved in 90 mL of dried THF and the insoluble solids remaining were filtered using a 1.2 μm glass membrane and discarded. The clear brownish solution remaining was evaporated under reduced pressure at room temperature and the wet solid obtained was dried in a heated dessicator under vacuum overnight. This dried solid (2.9503 g) was dissolved in 15 mL of DCE to be added to the polymer particles. The macroreticular particles (0.5210 g, 0.98 mmol chlorine/g) and DCE (15 mL) were added to a two-necked, round-bottomed flask fitted with a reflux condenser and a magnetic stirring bar. The particles were left to swell for 1 h under N_2. The solution of the tetra butylammonium salt of taurine in DCE prepared previously was then mixed with TEA (5 mL) and added to the flask. The mixture was stirred and heated at 80 °C for 18 h. After this period, the reaction mixture (brown colour) was cooled to room temperature

and the particles were filtered on a 0.2 μm nylon membrane filter. The particles PP-SCX/WAX were washed successively with DCE, THF, dichloromethane, ethyl acetate, MeOH, 1:1 MeOH:H_2O, H_2O, 2 M NaCl aqueous solution, 0.5 M HCl aqueous solution, 5% NH_4OH in MeOH solution, H_2O and acetone. The cream-colored particles were placed in a heated dessicator under vacuum overnight (0.5652 g).

2.2.3. PP-SCX/SAX

The same macroreticular particles prepared by PP and used for the synthesis of the PP-SCX/WAX sorbent were used for the development of the PP-SCX/SAX sorbent. These particles were functionalized using imidazole under basic conditions followed by a reaction with 1,3-propanesultone to introduce sulfonic acid groups into the polymer beads (Figure 3).

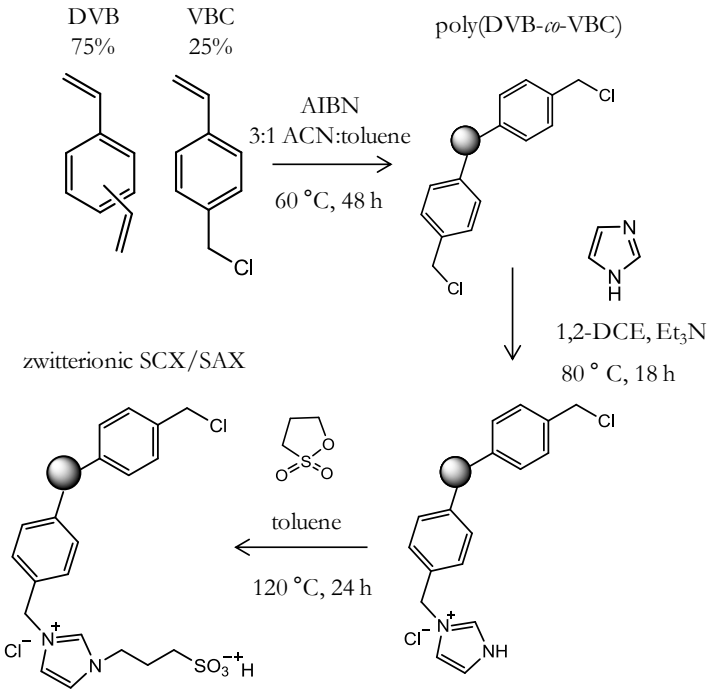

Figure 3. Schematic representation of the synthetic procedure used for the preparation of the PP-SCX/SAX sorbent.

For this purpose, the macroreticular particles (1.5044 g, 1.48 mmol of chlorine) were placed in a round-bottomed flask equipped with a reflux condenser, an overhead stirrer and an oil bath with controlled temperature. DCE (40 mL) was added and the particles were stirred (100 rpm) under N_2 at room temperature for 1 h. Imidazole (1.5141 g, 22.24 mmol) and TEA (9 mL) were dissolved in DCE (30 mL) and added to the reaction mixture before heating to 80 °C for 18 h. The particles were filtered (0.2 μm nylon membrane) and washed successively with DCE, toluene, EtOH, MeOH, 1:1 MeOH:H_2O, H_2O and acetone and then dried overnight in a vacuum oven (70 °C, 60 mbar) (1.5431 g). Subsequently, the particles functionalized with the imidazole groups (1.2060 g) were placed in a round-bottomed flask equipped with a reflux condenser, an overhead stirrer and an oil bath with controlled temperature. Dry toluene (15 mL) was added and the particles were stirred (100 rpm) under N_2 at room temperature for 1 h. 1,3-Propanesultone (1.5 mL, 2.088 g, 17.1 mmol) was added to the reaction mixture before heating to 120 °C for 24 h. The light brown coloured-particles were filtered (0.2 μm nylon membrane filter) and washed successively with toluene, EtOH and acetone and then dried overnight in a vacuum oven at 70 °C/60 mbar (1.2222 g).

2.2.4. Characterization

All of the sorbents synthesized were characterized using elemental microanalysis, Fourier-Transform infrared (FT-IR) spectroscopy, SEM and nitrogen sorption analysis. The carbon, hydrogen, nitrogen and sulfur contents were measured using a Perkin Elmer 2400 Series II CHNS Analyser while the chlorine content was determined by standard titration methods. FT-IR spectra were acquired using an Agilent Technologies 5500 Series Compact FT-IR instrument with a scanning range of 4,000 – 650 cm^{-1} in ATR mode. SEM was performed using a Cambridge Instrument Stereoscan 90 while a Polaron SC500A sputter coater was used to sputter-coat the samples in gold. The mean particle diameter was estimated by calculating the mean value of the diameters of a selection of 100 particles measured manually using ImageJ software [29] on SEM images. The porosity measurements were performed using a Micromeritics ASAP 2000 based on the N_2 sorption isotherm data provided by the instrument.

2.3. Solid-phase extraction procedure

100 mg of each of the synthesized sorbents were packed into 6 mL empty SPE cartridges (Symta, Madrid, Spain). A 10 μm polyethylene frit (also supplied by Symta) was placed at the bottom of the cartridge with a 2 μm stainless steel frit (Sigma-Aldrich) directly on top, followed by the sorbent and a second 10 μm polyethylene frit at the top of the sorbent bed. For all the extractions, the cartridges were placed in an SPE manifold (Teknokroma, Barcelona, Spain) connected to a vacuum pump to allow the percolation of liquids through the sorbents. The typical SPE protocol used started with the conditioning of the sorbents by passing 10 mL of MeOH through the cartridges followed by 10 mL of ultrapure water adjusted to the same pH as the sample. A 50 mL volume of ultrapure water adjusted to the selected pH (2, 5, 7 or 10) was spiked with the analytes at 0.7 mg/L (pharmaceuticals) or 1 mg/L (sweeteners and illicit drugs), and percolated through the sorbents at an approximate flow-rate of 5 mL/min. The washing step consisted of two fractions of 1 mL of MeOH which were collected independently and taken to a final volume of 5 mL with ultrapure water. The elution step was also performed in two fractions: 1) 5 mL of a 5% HCOOH in MeOH solution, followed by 2) 5 mL of a 5% NH_4OH in MeOH solution, which were collected individually and taken to a final volume of 25 mL with ultrapure water. As indicated, organic solutions were diluted to match with the starting mobile phase in the LC instrument to avoid distortion of the peak shapes. All fractions were injected directly to the chromatographic system, except for the basic solutions which were neutralized with concentrated HCOOH.

2.4. Instrumentation and chromatographic conditions

For the chromatographic analyses, an Agilent Technologies 1200 Series LC system was used, equipped with an automatic injector, a quaternary pump, a column oven and a DAD detector. The chromatographic columns used were an Ascentis Express C_{18} 100 x 4.6 mm, 2.7 μm for the separation of the pharmaceuticals and the illicit drugs, and a Tracer Excel 120 C_8 150 x 4.6 mm, 5 μm for the separation of the artificial sweeteners. For the analysis of the three groups of compounds, the mobile phase consisted of a mixture of solvent A (ultrapure

water adjusted to pH 2.8 using HCl) and solvent B (ACN). The oven temperature was set at 25 °C for both columns and the injection volume used was 25 μL in every case. The flow-rate was 0.4 mL/min for the separation of the pharmaceuticals, 0.5 mL/min to separate the illicit drugs and 0.6 mL/min in the case of the artificial sweeteners. The gradient profiles were also different for each group of compounds, as detailed in Table S4. All the pharmaceuticals were quantified at 210 nm with the exception of RAN that showed its highest signal at 310 nm. In the case of the drugs of abuse, BE, MDPV and COC were detected better at 230 nm while the rest were detected better at 210 nm. Most of the artificial sweeteners were detected with higher signals at 210 nm, with the exception of ACE (225 nm) and GLY (250 nm).

3 Results and discussion

3.1. Synthesis of the zwitterionic sorbents

Precipitation polymerization was the synthesis method selected for the preparation of the polymer particles because several studies [24, 30, 31] have demonstrated that this method yields high quality polymer microspheres with narrow particle size distributions and high specific surface areas. The specific surface areas are particularly high when the polymer microspheres are hyper-crosslinked. The use of a functional vinyl monomer, in this case VBC, and a crosslinking monomer such as DVB, produces highly crosslinked micron sized beads, with properties which depend upon the ratio of the monomers used during the synthesis as well as the polymerization conditions, and a tuneable content of functional groups derived from the functional vinyl monomer. In the case of VBC, the polymer microspheres are decorated with pendent chloromethyl groups that can be exploited in hypercrosslinking reactions and/or polymer analogous reactions. In the present study, two different monomer ratios were used to produce either gel-type particles (25/75, w/w, DVB/VBC feed ratio) or macroreticular particles (75/25, w/w, DVB/VBC feed ratio) with different chloride contents and specific surface areas. Gel-type particles are non-porous in the dry state but swell in thermodynamically compatible solvents, and it is for this reason that the gel-type particles were subjected to a Friedel-Crafts

reaction in the swollen state to obtain hypercrosslinked particles with high specific surface area. Macroreticular particles have permanent porosity, even in the dry state, and can be functionalized directly even in the presence of thermodynamically non-swelling solvents. The elemental microanalysis and FT-IR spectra of both types of particles confirmed the incorporation of both comonomers into the beads. The macroreticular particles contained 3.5 wt.% of chlorine whereas the hypercrosslinked particles had 7.9 wt.% chlorine, just as expected according to the DVB/VBC feed ratio used in each case. Characteristic bands were observed in the FT-IR spectra corresponding to the presence of chloromethyl groups in the particles, such as the Ar-CH_2-Cl wag at 1265 cm^{-1} and the C-Cl stretching vibration at 677 cm^{-1}. Particle size and size distribution were as expected from previous experience, and the particles were considered to be very suitable for SPE applications because they had low particle size and narrow particle size distributions, features that are important for efficient packing of the particles into cartridges and their performance as SPE sorbents. Figure S1 and S2 shows SEM images of the obtained particles.

The porous polymer microspheres were functionalized in a series of post-polymerization chemical modification reactions, based primarily on nucleophilic substitution reactions involving pendent chloromethyl groups. Amination reagents with the desired functionality were brought into contact with the polymer particles in a suitable solvent and under basic conditions. In the case of the PP-SCX/WAX sorbent, both ion-exchange groups were introduced into the polymer at the same time because the reagent used for the polymer functionalization reaction (taurine) had both functional groups present in an unprotected form (Figure 2). For the other two sorbents, the particles were firstly aminated and then subjected to subsequent reactions to introduce the cation-exchange character. The HXLPP-WCX/WAX sorbent was obtained from the reaction of the chloromethyl-containing particles with sarcosine ethyl ester hydrochloride under aqueous basic conditions (Figure 1). The final product contained tertiary amine and ester moieties. Subsequent hydrolysis of the ester groups to carboxylic acid groups resulted in a sorbent with zwitterionic properties. Elemental micro-analysis and FT-IR spectroscopic results supported the successful

modification of the particles. The nitrogen content of the final product increased by 0.5 % compared to the chloromethyl-containing polymer precursor and the chlorine content fell. Characteristic bands appeared in the FT-IR spectrum of the final product, such as the asymmetric $-CO_2^-$ stretch at 1602 cm^{-1} , while some bands decreased in intensity compared to the starting polymer such as the Ar-CH_2-Cl wag (1265 cm^{-1}), C-Cl stretching vibration (677 cm^{-1}), C=O stretch from the ester groups (1714 cm^{-1}) and C-O ester stretch (1184 cm^{-1}). Other properties of the sorbent are detailed in Table 1.

The synthesis of the PP-SCX/WAX sorbent was attempted under the aqueous basic conditions used for the synthesis of the HXLPP-WCX/WAX sorbent because taurine is soluble in water and insoluble in organic solvents. However, the elemental microanalysis and FT-IR spectroscopic data of the product suggested that the sorbent had a low level of functionalization. This was confirmed in preliminary SPE tests which showed that analytes could not be retained by the sorbent. For this reason, the tetrabutylammonium (TBA) salt of taurine was prepared as described in Section 2.2.2, and then used as the nucleophile under non-aqueous conditions because it is soluble in organic solvents and a better nucleophile than taurine [32]. Therefore, the substitution reaction was performed in DCE by bringing the chloromethyl-containing particles together with the taurine TBA salt and in the presence of TEA to provide the basic conditions (Figure 2). In this case, elemental microanalysis and FT-IR data showed that the functionalization of the particles was indeed successful (Table 1). New bands appeared in the FT-IR spectrum that could be assigned to the amine group (N-H stretch at 3300 cm^{-1}) and sulfonic acid group (1172 and 1036 cm^{-1}). This sorbent was very promising because it could be synthesized in two steps only and had a high level of functionalization (1.3 mmol N/g).

In the case of the PP-SCX/SAX sorbent, the imidazole unit was introduced first through the substitution of the chloromethyl

Table 1. Properties of the zwitterionic sorbents.

Polymer	Functional groups present	Microanalisis			Funct group loading (mmol/g)	Mean particle diameter (µm)	Specific surface area (m^2/g)
		N (%)	S (%)	Cl (%)			
HXLPP-WCX/WAX	carboxylic acid/ tertiary amine	0.9	-	2.6	0.6	3.8	850
PP-SCX/WAX	sulfonic acid/ secondary amine	1.8	0.9	6.8	1.3(N)/0.3(S)	6.6	**~2**
PP-SCX/SAX	sulfonic acid/ imidazolium	1.9	2.1	0.9	1.4(N)/0.7(S)	6.3	**~2**

groups and the product then treated with 1,3-propanesultone to introduce the sulfonic acid moieties [33] (Figure 3). Evidence for a successful functionalization was obtained by elemental microanalysis and FT-IR spectroscopic analysis. The final product contained 1.9 % of nitrogen and 2.1 % of sulfur while the chlorine content was reduced yet further after the modification (Table 1). Similar bands to those observed for the PP-SCX/WAX polymer were noted, attributable to the amine group (N-H stretch at 3300 cm^{-1}) and sulfonic acid group (1169 and 1037 cm^{-1}). Table 1 also shows the specific surface areas of the synthetized polymers. The high value obtained for the HXLPP-WCX/WAX particles is indicative of well-developed pore structures. However, the values for the PP-WCX/SAX and PP-SCX/SAX sorbents were very low. Differences concerning their SPE performance might be expected due to lower porosity.

3.2. SPE performance of the sorbents

The zwitterionic sorbents were evaluated by extracting three groups of analytes selected as model compounds, using in all cases the same SPE protocol as described in Section 2.3. The loading of the samples was tested at different pH values (pH 2, 5, 7 and 10) to evaluate the effect of this important parameter on the retention of the analytes and to provide insights into the properties and retention mechanisms of the sorbents. Figure 4 shows a diagram illustrating the charge form of the synthesized sorbents along the scale of pH. A MeOH washing step was included to evaluate which analytes were retained by hydrophobic interactions only as opposed to those analytes retained strongly by ionic interactions. The elution step also yielded important information with regards to the ionic interactions between sorbents and analytes, because depending on the pK_a values of the analytes and the functional groups of the sorbents, elution should be observed either in the acidic or the basic fraction.

The groups of model compounds selected include compounds with basic, neutral and acidic properties (see pK_a values in Table S1) that should promote different interactions with the sorbents. The group of illicit drugs includes basic compounds with less functional groups different from the amine functionalities, in contrary to basic pharmaceuticals, while the artificial sweeteners tested include

amino acids, acidic analytes (some with strong character) and neutral compounds with high polarity. It can be seen that the list of analytes comprises a high number of functional groups that form a very complex system when interacting with the zwitterionic sorbents. For this reason, the elucidation of the retention mechanisms present during the extraction for each analyte was very difficult, Therefore, the results obtained during the analytical evaluation will be discussed in terms of the trends observed in the retention of the analytes on each sorbent. The results are summarized in Tables 2, 3 and 4, where a selection of the most representative compounds is included.

3.3.1. HXLPP-WCX/WAX

When testing the extraction of the model compounds on the polymer with carboxylic acid and tertiary amine groups, it was observed that the neutral compounds CAFF, ANTI and CBZ were retained completely during the loading step and rinsed away to a high extent (~80 %) during the washing step.

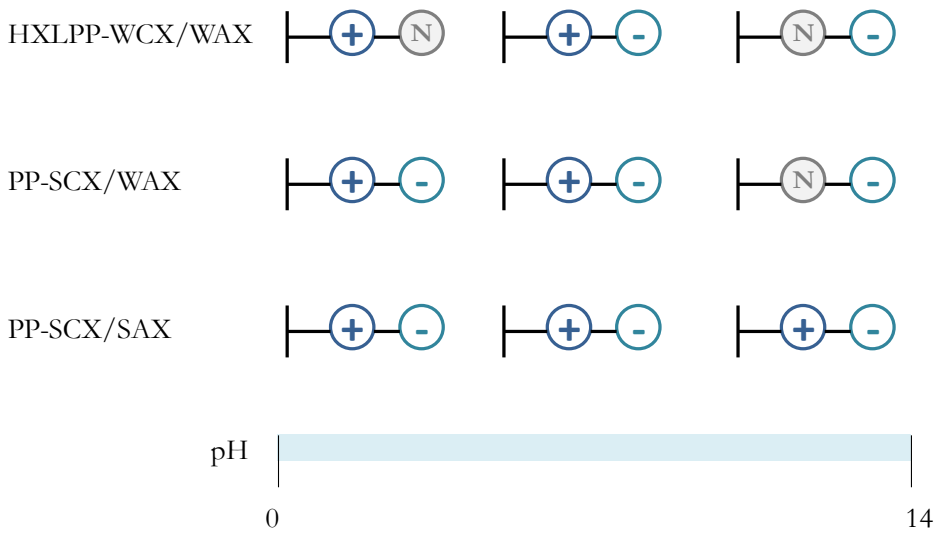

Figure 4. Diagram of the charge form of the zwitterionic sorbents along the pH scale.

It was also observed that changing the loading pH did not have any effect on the retention pattern of these analytes. Both observations suggested that these analytes were retained by hydrophobic interactions, as was expected. Similarly, NHDC did not seem to be affected by changes in the loading pH as it was always lost during the washing step with the same recoveries, results that agree with its neutral character.

The HXLPP-WCX/WAX sorbent will have zwitterionic, cationic or anionic character under certain pH conditions, depending on the pK_a values of the functional groups attached to the sorbent. At pH 2, the carboxylic groups within the sorbent would probably be in their neutral form while the amine groups would more likely be neutral at pH 12. Intermediate pH values are in theory the best conditions for having both types of moieties in their charged form.

In Table 2, the recoveries obtained for all of the SPE fractions of the HXLPP-WCX/WAX sorbent are shown for the different pH values tested, where w1 and w2 are the two 1 mL fractions of MeOH corresponding to the washing step, and e_a and e_b are the subsequent acidic and basic elution fractions (Section 2.3), respectively. In the case of compounds with weakly acidic properties, partial losses were observed in the washing fraction and, depending on the loading pH, some analytes were even lost in the loading step. For instance, 75 % of CLO AC was lost in the washing fraction when loading the sample at pH 2, while at pH 10 the analyte was almost fully lost (82 %) during the loading step, experiencing repulsion by the sorbent. DICLO was not lost during the loading step at any pH value but the recovery in the washing step increased as the pH increased. In general, the retention of the weakly acidic pharmaceuticals decreased with increasing pH, an observation that was not in agreement with the expected trend. These analytes should show poor retention at pH 2 because they are in their neutral state at this pH value, and also at pH 10 because the amine groups in the sorbent could also be deprotonated. However, the behaviour did not follow the pK_a values and losses in the washing fraction suggested the predominance of hydrophobic interactions over ionic ones. Because changes in pH were shown to have an impact on retention, it cannot be said that ionic interactions are not present,

Table 2. Recoveries obtained for some of the analytes selected, at each step of the SPE procedure and at different pH values, when using the HPLXPP-WCX/WAX sorbent.

	Analyte	Fraction	pH2	pH5	pH7	pH10
weak acid	**CLO AC**	L			18	82
	pK_a=3.4	w1	32	47	52	12
		w2	43	34	13	3
		e_a	20	14	11	0
		e_b				
		Σ	*95*	*95*	*94*	*97*
	DICLO	L				
	pK_a=4.0	w1	10	9	39	57
		w2	27	39	36	29
		e_a	52	41	18	9
		e_b				
		Σ	*89*	*89*	*93*	*95*
strong acid	**ACE**	L		39	92	89
	pK_a=-0.3	w1		7	2	
		w2		3	1	
		e_a		1		
		e_b	88	23		
		Σ	*88*	*73*	*95*	*89*
amino acid	**ALI**	L				76
	pK_a=3.4, 8.2	w1	59	42	35	12
		w2	15	19	23	3
		e_a	2	13	2	1
		e_b		3	10	
		Σ	*76*	*77*	*70*	*91*

Table 2. (Cont.)

	Analyte	Fraction	pH2	pH5	pH7	pH10
weak acid	**NHDC**	L				
	pK_a=6.8	w1	28	23	17	21
		w2	24	21	29	20
		e_a	31	37	42	29
		e_b	1	1	6	12
		Σ	*84*	*82*	*93*	*83*
weak base (pharmaceuticals)	**ATE**	L				
	pK_a=9.7	w1	56	32	25	0
		w2	13	27	12	0
		e_a	0	16	61	97
		e_b				
		Σ	*69*	*75*	*98*	*97*
	PROP	L				
	pK_a=9.7	w1	53	9	7	0
		w2	39	10	4	0
		e_a	8	79	91	96
		e_b				
		Σ	*100*	*98*	*102*	*96*
weak base (illicit drugs)	**COD**	L				
	pK_a=9.2	w1	63			1
		w2	24			9
		e_a	12	89	84	82
		e_b		2		
		Σ	*99*	*91*	*84*	*92*
	BE	L				
	pK_a=9.5	w1	37	28	13	26
		w2	31	36	41	37
		e_a	30	32	39	39
		e_b				
		Σ	*98*	*96*	*93*	*102*

but they are surely not predominant in this case.

In the case of compounds with relatively strong acidic properties, strong ionic interactions were observed for ACE, SAC and GLY only when loading the sample at pH 2 for ACE and up to pH 5 for SAC and GLY. For the rest of the pH values these analytes were rinsed during the loading step.

This observation suggests that this sorbent can be quite selective for strong acids. With regards to the weakly basic compounds, no losses were observed during the loading step but losses were noticeable during the washing step. In this case, the retention increased with increasing pH in constratst to the weakly acidic compounds. For instance, in Table 2 ATE was lost completely in the washing step (69 % as total of the two washing fractions) as expected when working at pH 2, but it was not lost when loading at pH 10, showing a recovery of 97 % during the acidic elution. The remainder of the weakly basic compounds (basic pharmaceuticals) behaved similarly, showing that the sorbent interacted strongly with these compounds through ionic interactions when loading the sample at pH 10. At this pH value, the carboxylic acid groups of the sorbents should be in their anionic form ready to retain positively charged compounds, but in this case the analytes were very close to their pK_a values, so there is a high probability that analytes are only partially charged. Certainly, it was not expected that retention would be lower at intermediate pH values such as 5 or 7 where both the analytes and the sorbents should be charged. The presence of several functional groups in the analytes apart from the amine groups could be responsible for introducing other retention mechanisms that might explain the retention behaviour.

In comparison, results that were in better agreement with the pK_a of the analytes and the sorbents were observed when evaluating the basic compounds with different structures and less contributions from other functional groups. In Table 2, it can be seen how COD was lost during the washing step (87 %) when loading the sample at pH 2 while for the rest of the pH values the analyte was retained ionically, being recovered in the acidic elution (82 – 89 %). The rest of these bases (illicit drugs) behaved similarly, except for BE which was lost partially during the washing step at all pH values, proving to be unaffected by

changes to this parameter. The presence of the carboxylic acid group near the amine group in the structure of BE might be responsible for this behaviour. The lone pair of the amine might prefer to form an intermolecular hydrogen bond with the carboxylic acid group. Alternatively, there might be repulsive interactions between the carboxylic acid group of the analyte and the negatively charged moieties of the sorbent. In any case, the sorbent seems to be more selective for bases with the structural properties of the selected illicit drugs than for bases with structures like the pharmaceuticals.

In the case of ASP, ALI and NEO, which show properties typical of amino acids, the behaviour was quite complex. In general, they were not retained strongly, but ionic interactions were present because the loading pH affected their retention. As an example, it can be seen in Table 2 that ALI showed higher retention at pH 5 and 7 when compared to pH 2 or 10, but for all the pH values the analyte was almost lost completely during the washing step. So far, it has been observed that this sorbent does not retain relatively weak acids but is quite selective for strong acids. An explanation for this behavior might be that the amine groups of the sorbent have weak ion-exchange properties due to the influence of the nearby carboxylic acid groups. Tests performed on the amino ester sorbent prior to the introduction of the carboxylic groups via ester hydrolysis supports this hypothesis (data not shown). Therefore, a synergy between both ionizable groups in the zwitterionic sorbent can be argued, which confers the polymer with very interesting and unusual properties. The HXLPP-WCX/WAX sorbent has shown potential to simultaneously and selectively extract acidic compounds with strong character and basic analytes with certain structures.

3.3.2. PP-SCX/WAX

On this sorbent, the neutral compounds CAFF, ANTI and CBZ behaved similarly to the previous sorbent in that they were retained completely during the loading step but rinsed off to a great extent (~90 %) during the washing step. In contrast, NHDC was retained strongly at pH 10 (Table 3) which can be explained by the weakly acidic character of this analyte.

Weakly acidic compounds showed strong retention on the sorbent

Table 3. Recoveries obtained for some of the analytes selected, at each step of the SPE procedure and at different pH values, when using the PP-SCX/WAX sorbent.

	Analyte	Fraction	pH2	pH5	pH7	pH10
weak acid	**CLO AC**	L				
	pK_a=3.4	w1	7			
		w2	10			
		e_a	69	89	89	93
		e_b				
		Σ	*86*	*89*	*89*	*93*
	DICLO	L				
	pK_a=4.0	w1	18			
		w2	5			
		e_a	76	92	89	92
		e_b				
		Σ	*99*	*92*	*89*	*92*
strong acid	**ACE**	L				
	pK_a=-0.3	w1				
		w2				
		e_a				
		e_b				21
		Σ	*0*	*0*	*0*	*21*
amino acid	**ALI**	L	79			
	pK_a=3.4, 8.2	w1	5			
		w2				
		e_a		73	74	84
		e_b			7	5
		Σ	*84*	*73*	*81*	*89*

Table 3. (Cont.).

	Analyte	Fraction	pH2	pH5	pH7	pH10
neutral/weak acid	**NHDC**	L				
	pK_a=6.8	w1	77	2	6	
		w2	11	22	14	
		e_a	3	60	57	82
		e_b			3	
		Σ	*91*	*84*	*80*	*82*
weak base (pharmaceuticals)	**ATE**	L	79	74	75	
	pK_a=9.7	w1				44
		w2				17
		e_a				
		e_b				
		Σ	*79*	*74*	*75*	*60*
	PROP	L			17	
	pK_a=9.7	w1	47	44	35	45
		w2	19	34	28	44
		e_a	21	11		3
		e_b				
		Σ	*87*	*89*	*80*	*92*
weak base (illicit drugs)	**COD**	L	92	77	62	
	pK_a=9.2	w1	2	14	24	59
		w2	1	1	4	24
		e_a				4
		e_b				
		Σ	*95*	*92*	*90*	*87*
	BE	L	23	6	6	
	pK_a=9.5	w1	40	65	44	24
		w2	30	24	33	27
		e_a	2	2	14	63
		e_b				
		Σ	*95*	*97*	*97*	*114*

for almost all of the pH values and no losses were observed during the washing step. Only at pH 2 some of the analytes were lost partially, which was in agreement with their pK_a values (Table 3). When testing the more strongly acidic compounds, the capability of the sorbent for retaining them was confirmed further, as all of the analytes were retained strongly on the polymer. In fact, ACE and SAC, which have strongly acidic character, were very strongly retained, so much so that forcing conditions (15 % HCOOH in MeOH) were required for their complete elution. This strong retention was observed at all pH values for ACE and SAC, and started from pH 5 for ASP, ALI, NEO and GLY which have amino acid-like properties. Indeed, ASP and ALI were rinsed during the loading step when working at pH 2. This behavior was in agreement with their pK_a values showing the great versatility of the sorbent for retaining all types of acidic compounds.

Surprisingly, the weakly basic compounds were retained poorly; they were rinsed completely during the loading and the washing steps, depending on the loading pH. For instance, in Table 3 ATE was lost during the loading step when using pH 2, 5 and 7 for loading the sample and rinsed during the washing step when loading the sample at pH 10. PROP was barely lost during the loading step but it was rinsed off to a great extent (~75 %) during the washing step. The lack of retention on the sorbent for weakly basic compounds suggests that the sulfonic acid groups were not accessible to the analytes. The same behavior was observed when extracting the group of basic compounds with different structures (illicit drugs). At all the pH values tested to load the sample, the basic analytes were lost during the loading or the washing step. Differences among these pH values were observed as retention tended to increase when increasing the pH. This behavior does not agree with the expected properties of the sorbent and the analytes because the sulfonic acid groups present should be charged at all pH values and analytes are neutral from pH ~ 7 to 9. Therefore, retention should be similar at pH 2, 5 and 7 but should decrease at pH 10. The same analytes have been retained successfully on sorbents bearing only sulfonic acid groups [23, 34]. Possibly, there are other retention mechanisms present apart from hydrophobic or ionic interactions affecting the retention behavior. As discussed before, the

functionalization of the particles was indeed successful because acidic compounds were retained strongly and characterization data suggested the presence of functional groups containing sulfur. The lower porosity obtained for this sorbent could be responsible for the poor accessibility to the sulfonic groups.

3.3.3. PP-SCX/SAX

The results obtained for the PP-SCX/SAX sorbent were very similar to those observed for the PP-SCX/WAX sorbent. Neutral compounds were rinsed completely (~95 %) during the washing step and were unaffected when loading at different pH values, suggesting that they were retained through hydrophobic interactions alone. In general, all acidic compounds were retained strongly at pH 5, 7 and 10. At pH 2, some weakly acidic compounds were lost partially during the washing step, especially in the case of IBP which was retained better at pH 7 and 10, both observations being consistent with the pK_a values of the analytes. Once again, the results supported the characterization data confirming the successful functionalization of the particles. Nevertheless, the retention behavior of the basic compounds indicated that the sulfonic groups were inaccessible to the analytes. All basic compounds (pharmaceuticals and illicit drugs) were lost during the washing and the loading steps. For instance, in Table 4 ATE was lost during the loading step when using pH 2, 5 and 7 for loading the sample and was rinsed during the washing step when loading the sample at pH 10. PROP was retained during the loading step but it was rinsed off the sorbent to a considerable extent (~65 %) during the washing step. It was also observed that the change of pH had an effect on the retention behavior, because retention tended to increase when increasing the pH, a trend which is not in agreement with the pK_a values of the compounds. Possibly, other retention mechanisms like hydrogen bonding were contributing apart from hydrophobic or ionic interactions affecting the retention behavior.

Given that the preparation of the functionalized sorbent was performed in two steps, firstly the substitution of the chloride groups for the imidazole groups and secondly the introduction of the sulfonic groups by the reaction with 1,3-propanesultone, it can be argued that the second reaction did not go to completion meaning that there are more anion-exchan-

Table 4. Recoveries obtained for some of the analytes selected, at each step of the SPE procedure and at different pH values, when using the PP-SCX/SAX sorbent.

	Analyte	Fraction	pH2	pH5	pH7	pH10
weak acid	**CLO AC**	L				
	pK_a=3.4	w1	12		2	
		w2	9		1	
		e_a	81	99	97	101
		e_b				
		Σ	*102*	*99*	*100*	*101*
	DICLO	L				
	pK_a=4.0	w1	13			
		w2	7			
		e_a	73	101	99	100
		e_b				
		Σ	*93*	*101*	*99*	*100*
strong acid	**ACE**	L				
	pK_a=-0.3	w1				
		w2				
		e_a				
		e_b			3	17
		Σ	*0*	*0*	*3*	*17*
amino acid	**ALI**	L	76			
	pK_a=3.4, 8.2	w1	3			
		w2		1		
		e_a		64	60	84
		e_b			17	6
		Σ	*79*	*65*	*77*	*90*

Table 4. (Cont.).

	Analyte	Fraction	pH2	pH5	pH7	pH10
neutral/weak acid	**NHDC**	L				
	pK_a=6.8	w1	59	4	1	
		w2	19	7	1	
		e_a	10	74	65	91
		e_b			1	
		Σ	*88*	*85*	*68*	*91*
weak base (pharmaceuticals)	**ATE**	L	83	81	66	
	pK_a=9.7	w1		1	5	37
		w2			2	16
		e_a				
		e_b				
		Σ	*83*	*82*	*73*	*53*
	PROP	L	2	7		
	pK_a=9.7	w1	57	48	43	41
		w2	25	14	13	18
		e_a	11	16	29	27
		e_b	3	2	4	7
		Σ	*98*	*87*	*89*	*93*
weak base (illicit drugs)	**COD**	L	84	52	28	
	pK_a=9.2	w1	3	22	36	48
		w2	2	6	10	18
		e_a		4	6	12
		e_b				3
		Σ	*89*	*84*	*80*	*81*
	BE	L	14	4		
	pK_a=9.5	w1	34	30	27	15
		w2	34	37	39	11
		e_a	10	24	25	83
		e_b				
		Σ	*92*	*95*	*91*	*109*

ge groups than cation-exchange groups [33]. If only a fraction of imidazole groups reacted with the 1,3-propanesultone leaving some unreacted groups, then the net charge of the sorbent could be positive, causing repulsive forces on basic compounds. In an earlier study, differences in retention were observed between sorbents having the same ion-exchange capacity for positively and negatively charged groups in comparison with sorbents having more cation-exchange groups (an excess of negative charges) [27]. However, even if the sorbent has more anion-exchange groups than cation-exchange groups, the retention behavior was very similar to the PP-SCX/WAX sorbent, which has the same number of cation- and anion-exchange groups as both moieties were introduced into the polymer at the same time.

4 Conclusions

The synthesis and application for SPE purposes of three types of polymeric mixed-mode sorbents containing zwitterionic moieties is evaluated; a SPE strategy that has not been thoroughly explored as described herein. Polymer characterization data showed the successful synthesis of the desired sorbents and the simultaneous incorporation of cation-exchange and anion-exchange moieties within the sorbents.

The SPE evaluation of the three sorbents performed using a list of analytes selected for their basic, neutral and acidic properties gave valuable insights into the properties of the sorbents. The HXLPP-WCX/WAX sorbent synthesized from sarcosine ethyl ester proved to be very selective for ionic compounds with strong properties, especially analytes with strongly acidic character. A synergy between the amine and carboxylic acid groups of the zwitterionic moiety was observed, in that the presence of the carboxylic acid group seemed to lower the pK_a value of the adjacent amine group. Both the PP-SCX/WAX and PP-SCX/SAX sorbents behaved very similarly, which was surprising because they had distinct chemical structures. Both sorbents exhibited a great capacity for retaining a broad range of acidic compounds, but they showed poor selectivity for basic compounds.

The introduction of zwitterionic mixed-mode polymers as SPE sorbents has been presented with promising results. Future research will be focused on understanding

the retention mechanisms during the SPE, to better exploit their selectivity.

Acknowledgments

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LC-MS/MS for the re-evaluation of free 3-nitrotyrosine in human plasma as an oxidative stress biomarker, Talanta 140 (2015) 45-51.

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chromatography tandem mass spectrometry detection, J. Pharm. Biomed. Anal. 54 (2011) 1136-1145.

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Supplementary Data

Table S1. Structures and pK_a values of the selected pharmaceuticals.

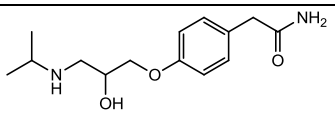
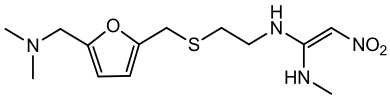
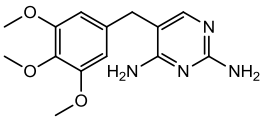
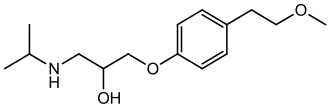
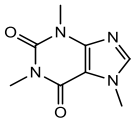
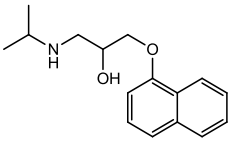
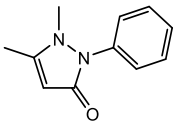
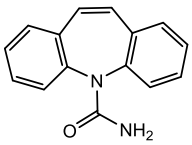
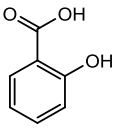
Compound	Structure	pK_a
Atenolol (ATE)		9.7
Ratinidine (RAN)		8.1
Trimetoprim (TRI)		5.3, 10.8
Metaprolol (MET)		9.7
Caffeine (CAFF)		-1.2
Propanolol (PROP)		9.7
Antipyrine (ANTI)		0.5
Carbamazepine (CBZ)		16.0
Salicilic acid (SAL AC)		2.8

Table S1. (Cont.).

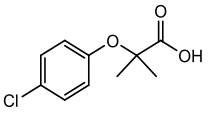
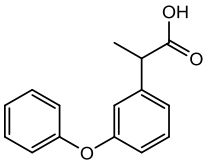
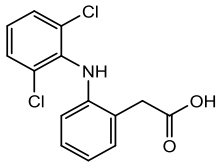
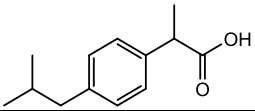
Compound	Structure	pK_a
Clofibric acid (CLO AC)		3.4
Fenoprofen (FEN)		3.9
Diclofenac (DICLO)		4.0
Ibuprofen (IBP)		4.9

Table S2. Structures and pK_a values of the selected illicit drugs.

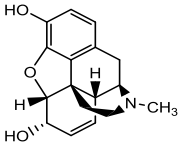
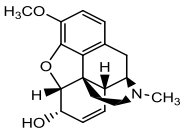
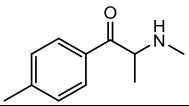
Compound	Structure	pKa
Morphine (MOR)		9.1
Codeine (COD)		9.2
Mephedrone (MMC)		8.0

Table S2. (Cont.).

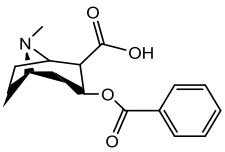
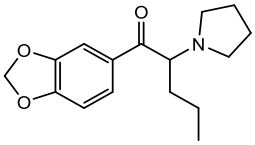
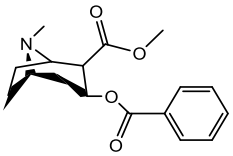
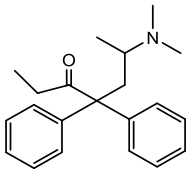
Compound	Structure	pKa
Benzoylecgonine (BE)		9.5
Methylene-dioxypyrvalerone (MDPV)		7.3
Cocaine (COC)		8.8
Methadone (METH)		9.1

Table S3. Structures and pK_a values of the selected artificial sweeteners.

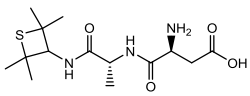
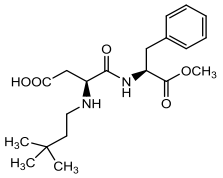
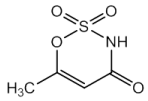
Compound	Structure	pK_a
Alitame (ALI)		3.4 (COOH) 8.2 (NH_2)
Neotame (NEO)		4.2 (COOH) 9.1 (NH_2)
Acesulfame (ACE)		-0.3

Table S3. (Cont.).

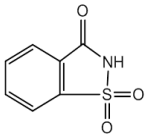
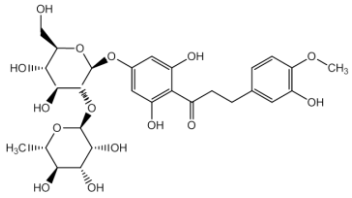
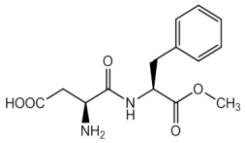
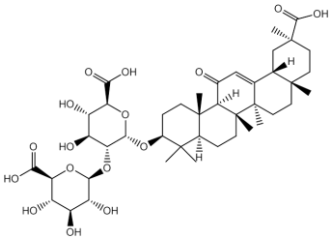
Compound	Structure	pKa
Saccharine (SAC)		1.6
Neohesperidine (NHDC)		6.8
Aspartame (ASP)		3.7
Glycyrrhizic acid (GLY)		2.8

Table S4. Gradient profiles used in the LC-DAD instrument for the separation of each group of EOCs.

Pharmaceuticals		Drugs of abuse		Artificial sweeteners	
t (min)	%B (ACN)	t (min)	%B (ACN)	t (min)	%B (ACN)
0	2	0	2	0	15
9	70	5	15	13	45
13	75	10	20	20	100
14	100	15	70	22	100
15	2	17	100	24	15
20	2	20	2		
		25	2		

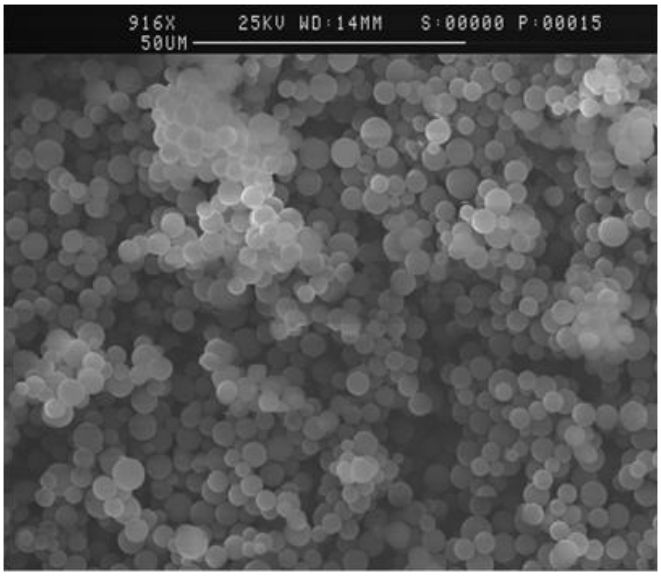


Figure S1. SEM image of the HXLPP-WCX/WAX sorbent.

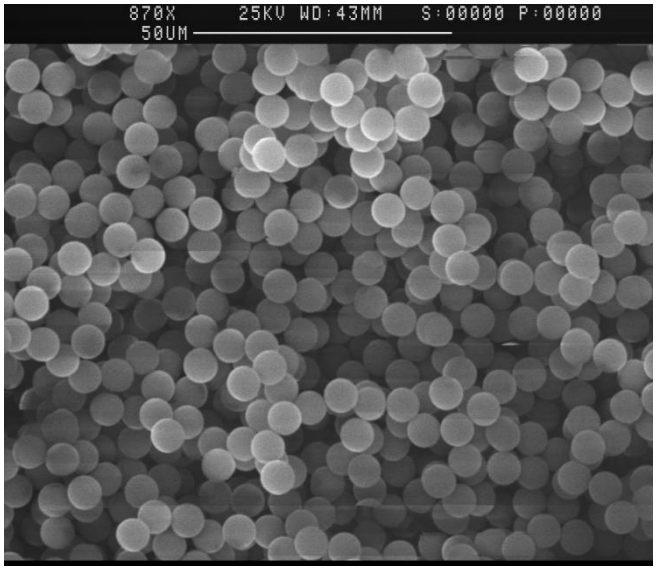


Figure S2. SEM image of the PP-SCX/SAX sorbent.

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3.3.2. Discussion of results

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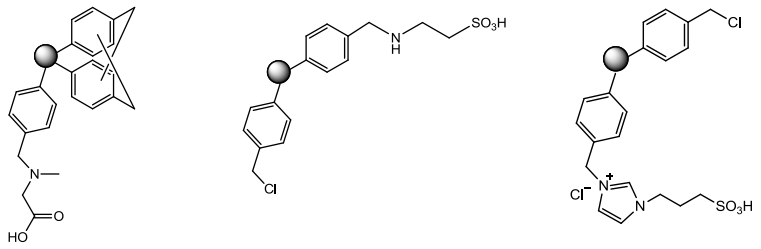
Three types of mixed-mode zwitterionic polymeric particles were prepared and evaluated as sorptive materials for solid-phase extraction (SPE). The synthesis of the particles and their functionalization with zwitterionic groups was described, together with their study as extracting materials of a group of pharmaceuticals, drugs of abuse and artificial sweeteners, selected as model compounds for their acidic and basic properties. Because detailed discussion of the results obtained in this study was provided, in this section the most important outcomes will be emphasized. The motivation for the development of these novel materials arose from the need to widen the capabilities of mixed-mode sorbents to simultaneously retain both basic and acidic compounds, rather than one single type of compounds. It was demonstrated in Section 3.2.3 of this Doctoral Thesis, that combining both positive and negative charges (anion- and cation-exchangers) in the same SPE cartridge allowed the simultaneous extraction of basic and acidic compounds using a simple protocol. In this section, the idea was to obtain sorbents with both charged groups in the same moiety and evaluate their performance and retention behavior for different types of compounds.

For the development of the functionalized sorbents, starting particles were subjected to post-polymerization modification reactions for the introduction of the zwitterionic functionality. These particles were of two types depending on the monomer feed ratio DVB/VBC (Table 1) during the polymerization, obtaining either gel-type or macroreticular particles. As previously stated, the gel-type particles were prepared with low DVB content to obtain a material with high chlorine content susceptible to HXL reactions to increase surface area from negligible to >1000 m^2/g. High surface areas of HXL materials is desirable in terms of sorbent capacity but, because the HXL reaction takes profit of the pending chloride groups, it can be detrimental in further functionalization reactions if not enough chloride groups remain available for modification. In this sense, macroreticular particles have a higher amount of chloride groups but less surface areas. Therefore, it becomes clear that a compromise between capacity and level of functionalization must be taken into account when developing these types of materials.

Table 1. Properties of unmodified polymeric particles.

Type of particle	Monomer ratio DVB/VBC (w/w)	Microanalysis				Surface area (m^2/g)
		C (%)	H (%)	N (%)	Cl (%)	
PP gel-type	25/75	77.3	6.5	0.3	12.8	< 1
HXLPP		84.7	6.9	0.5	7.9	1137
PP macroreticular	75/25	86.4	7.6	0.4	3.5	128

For these reasons, hypercrosslinked (HXL) and macroreticular particles were both potential starting material for the development of mixed-mode zwitterionic-exchange sorbents. In the case of the firstly introduced sorbent, containing tertiary amine and carboxylic acid groups in the same moiety (with weak cation- /anion-exchange WCX/WAX properties, Figure 1), HXL particles were used as starting material for functionalization. The development of this sorbent derived from the efforts of Anderson *et al.* [1] in the synthesis of materials with weak anion-exchange (WAX) properties using sarcosine ethyl esters. In this study, the WAX sorbent contained ethyl ester groups which included a novel feature for this type of materials and provided a route into zwitterionic sorbents. Because preliminary SPE studies showed potential for the selective extraction of acidic compounds, the continuation of this research was proposed in the present Thesis. However, to obtain higher amount of pending chlorides and thus highly functionalized sorbents, the reaction time of the HXL reaction was decreased from 18 h to 10 min in comparison to the previous procedure. This modification of the procedure was motivated by results obtained in the studies of Davies *et al.* [2] where it was demonstrated that HXL reaction proceeds very rapidly suggesting that, in order to leave behind enough chloromethyl groups, the reaction should progress for around 10 minutes.



HXLPP-WCX/WAX PP-SCX/WAX PP-SCX/SAX

Figure 1. Sorbents synthesized with zwitterionic character.

Probably because of the low DVB content of the gel-type particles, they showed a lower particle size and a wider particle size distribution when compared to macroreticular beads. As expected after the HXL reaction, there was an increase of specific surface area together with a decrease in chlorine content. However, the chlorine percentage was higher in this case compared with materials subjected to 18 h HXL reactions, leaving more chloride groups susceptible to modification reactions. As a result, the functionalized polymer in this case showed content in nitrogen of 0.9 % in comparison to previous sorbent [1] which had 0.6 %.

For the other zwitterionic-exchangers, macroreticular particles were used as starting material for functionalization. These beads obtained in one single step had suitable chlorine content (3.5%) to be directly functionalized after polymerization. However, the specific surface area obtained for this polymer was low (128 m^2/g) which might suggest that a higher volume of porogen (toluene) was required during polymerization, although this is not suitable for obtaining spherical particles in PP, where ACN is the preferred solvent [2]. The functionalized material containing secondary amine and sulfonic acid groups in the same moiety (with weak cation- /strong anion-exchange SCX/WAX properties) was synthesized using taurine as the amino reagent for the substitution reaction. As previously mentioned, the substitution reaction of the macroreticular particles with taurine was initially tested under aqueous conditions, by swelling the particles in ethanol and adding an aqueous solution of taurine and potassium carbonate. The reaction was

thought feasible because in the study by Aydogan *et al.* [3] a 0.6 M aqueous solution of taurine adjusted at pH 6.9 was passed through a monolith of poly(3-chloro-2-hydroxypropyl methacrylate-*co*-ethylene dimethacrylate) for its functionalization, to develop a zwitterionic electrochromatographic stationary phase. The results of microanalysis of the macroreticular particles before and after the substitution reaction are shown in Table 2. A decrease in chlorine content together with the increase of nitrogen content is evidence of the introduction of taurine groups into the polymer. However, the degree of functionalization seemed to be low which was later confirmed with the results of the SPE evaluation.

For this reason, another synthetic strategy was tested using the tetrabuthylammonium (TBA) salt of taurine which was obtained by mixing the dissolved taurine with tetrabuthylammonium hydroxide [4]. Because this salt is soluble in organic solvents in contrary to the crystalline taurine, it was possible to conduct the reaction in 1,2-dichloroetane which allows better swelling of the particles than water. Besides, the quaternary amine salt ammonium salt of taurine is better nucleophile than crystalline taurine [5]. Microanalysis (Table 2) and FT-IR data demonstrated that this synthetic strategy was more effective, producing in only two steps a polymeric material with zwitterionic moieties.

Table 2. Microanalysis data before and after modification reaction.

Type of particle	**Microanalysis**			
	C (%)	**H (%)**	**N (%)**	**Cl (%)**
PP macroreticular	86.4	7.6	0.4	3.5
PP-SCX/WAX particles modified using taurine	85.6	7.4	0.7	2.6
PP-SCX/WAX particles modified using the TBA salt of taurine	81.9	8.6	1.8	6.8

In the case of the sorbent containing imidazole and sulfonic acid groups (with strong cation- /anion-exchange SCX/SAX properties), the imidazole groups were firstly introduced in a substitution reaction using similar conditions as before, this is, in 1,2-dichloroetane under basic conditions given by an excess of triethylamine. This strategy was also successfully used to functionalize HXL particles based on VBC-DVB with EDA and piperazine [6]. In Table 3, microanalysis results are shown obtained for the functionalized particles in comparison with the unmodified ones.

Table 3. Microanalysis data before and after modification reactions.

Type of particle	**Microanalysis**				
	C (%)	**H (%)**	**N (%)**	**Cl (%)**	**S (%)**
PP macroreticular	86.4	7.6	0.4	3.5	-
PP-SAX particles modified using imidazol	82.8	7.7	2.0	2.7	-
PP-SCX/SAX particles containing imidazole groups modified using the 1,3-propanesultone	78.9	7.5	1.9	0.9	2.1

In this case, the nitrogen content significantly increased suggesting the introduction of the imidazole groups while the chlorine content slightly decreased, probably because part of this chlorine was now present as counterion of the imidazole groups rather as chloromethyl groups attached to the polymer. Following conditions as described in the study by Qiu *et al.* [7], the imidazole groups in the beads were reacted with 1,3-propanesultone because this heterocycle can undergo cleavage of their carbon−oxygen bond in the presence of nucleophiles and can therefore be used as sulfoalkylating agent. The reaction proved to be successful as FT-IR bands of sulfur containing groups were observed and the sulfur content was the expected. Figure 1 shows the FT-IR spectra obtained for the macroreticular particles before and after functionalization. In the spectrum of the functionalized

polymer, a decrease of peaks at 1266 (Ar-CH_2-Cl) and 686 (C-Cl) cm^{-1} is observed, as well as the apparition of peaks at ~3400 (N-H) and at 1206, 1169, 1113 and 1037 cm^{-1} (all attributable to sulfur containing groups), suggesting the successful substitution of chloride groups by the desired moiety.

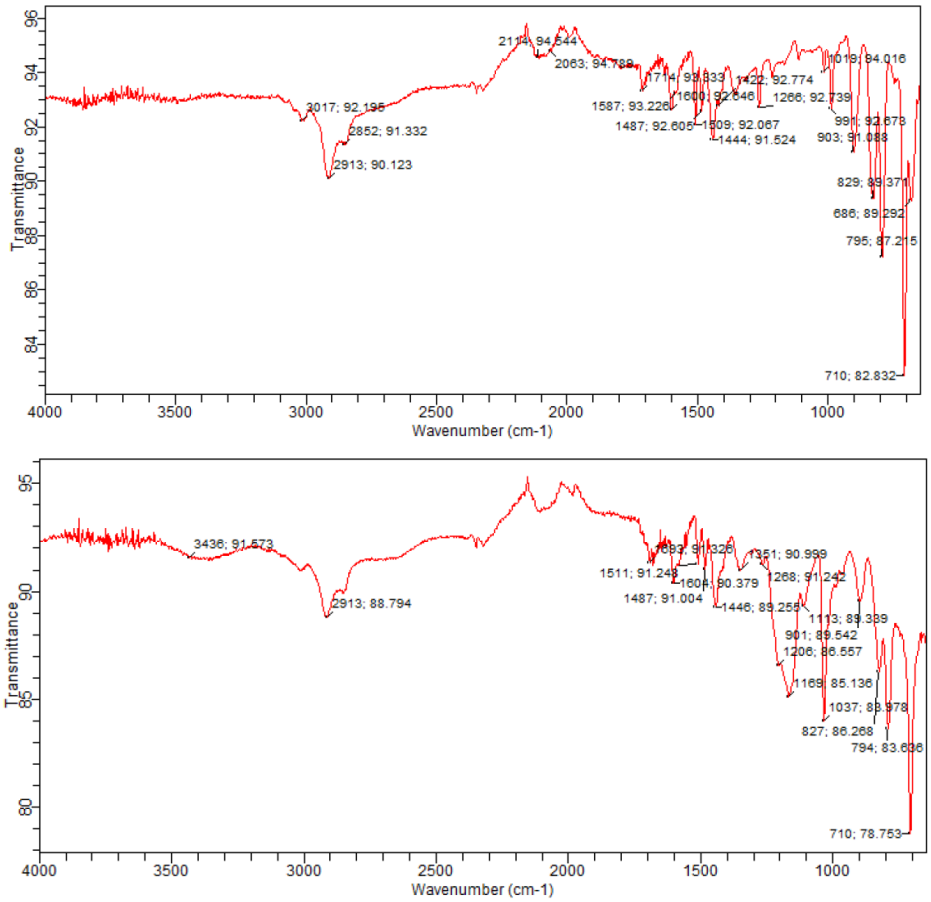


Figure 1. FT-IR spectra obtained for the starting macroreticular particle (up) and the PP-SCX/SAX sorbent (bottom).

Considering that characterization data suggested the successful functionalization of the polymers, they were evaluated as sorptive materials in different SPE tests. The extraction of a list of model compounds using the

same SPE procedure gave insights into the interactions promoted by the sorbents under different pH and solvent conditions. These compounds were selected according to their different structures and properties, including basic and acidic with weak and strong character. From the results of the SPE evaluation, it was observed that the HXLPP-WCX/WAX sorbent showed high selectivity for strong acids and bases with specific structures, as they established strong ionic interactions with some acidic sweeteners and the basic drugs of abuse selected, in opposition to other compounds with acidic and basic properties. Neutral compounds were only retained through hydrophobic interactions being rinsed during the methanol washing step, similar to acidic and basic compounds with weak properties and aminoacids, which were also washed away but the presence of additional ionic interferences were observed. From the three materials developed, this was the most promising one as zwitterionic-exchanger because the PP-SCX/WAX and PP-SCX/SAX materials were capable of selectively retaining any type of acidic compounds but incapable of ionically retaining basic compounds. The explanation for this behaviour might be the lack of porosity observed for the materials. In fact, functionalized polymers gave negligible specific surface areas (~2 m^2/g) in comparison to the starting material (128 m^2/g) suggesting that the introduction of the functional groups made the pore even less accessible. Nevertheless, the good recoveries obtained for neutral and acidic compounds also indicates that the sorbent has enough capacity to retain the analytes through reversed-phase interactions and that amine groups are available in the sorbent. It is odd that the only problem derived from the low porosity seems to be the inaccessibility of the sulfonic groups, as if they are highly hindered or spatially disposed in a form that the binding sites are not available for the compounds. Future research will be focused on this matter in order to improve the performance of these materials as zwitterionic-exchangers.

It was also noticeable how pharmaceuticals showed differences in the retention behavior when compared to combined sorbents in the previous Section 3.2.2. For example, diclofenac was ionically retained on the four configurations (SCX/SAX, SAX/WCX, SCX/WAX, WCX/WAX) obtained from mixing commercial sorbents. Likely, it was selectively retained on the

PP-SCX/WAX and PP-SCX/SAX in-house sorbents. However, it was partially lost during the washing step when retained on the HXLPP-WCX/WAX in-house sorbent. In this case, the influence of the weak and strong character of the functional groups is evident.

The results obtained in the present Thesis opens a promising path into the development of new sorbents with very interesting properties that could be exploited for several applications benefiting from their enhanced capacity and selectivity toward ionizable compounds.

References

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[2] A. Davies, Synthesis and characterisation of strong and weak ion-exchange materials, University of Strathclyde, Glasgow, Scotland, UK, 2009.

[3] C. Aydogan, B. Celebi, A. Bayraktar, F. Yilmaz, A. Denizli, A. Tuncel, A new zwitterionic electrochromatographic stationary phase based on poly(3-chloro-2-hydroxypropyl methacrylate-co-ethylene dimethacrylate) reactive monolith, Polym. Adv. Technol. 25 (2014) 777-783.

[4] D.G.I. Kingston, 1992, European Patent 0473326A1

[5] T. Ogata, T. Shimazaki, T. Umemoto, S. Kurata, T. Ohtsuki, T. Suzuki, T. Wada, Chemical Synthesis and Properties of 5-Taurinomethyluridine and 5-Taurinomethyl-2-thiouridine, J. Org. Chem. 74 (2009) 2585-2588.

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[7] H. Qiu, Q. Jiang, Z. Wei, X. Wang, X. Liu, S. Jiang, Preparation and evaluation of a silica-based 1-alkyl-3-(propyl-3-sulfonate) imidazolium zwitterionic stationary phase for high-performance liquid chromatography, J. Chromatogr. A 1163 (2007) 63-69.

CHAPTER 4. CONCLUSIONS

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The most important conclusions derived from the studies presented in this Doctoral Thesis can be outlined as follows:

1. The successful application of hydrophilic interaction liquid chromatography (HILIC) was demonstrated for the separation of polar and hydrophilic compounds relevant to the environmental field, proving that is a promising alternative technique for improving their chromatographic separation and determination.

2. Good chromatographic separation of iodinated X-ray contrast media and artificial sweeteners are possible using HILIC, being an alternative technique for these compounds little studied by this LC mode.

3. The use of HILIC provided enhanced retention and alternative selectivity compared to conventional reversed-phase liquid chromatography for the studied compounds.

4. Both stationary and mobile phase conditions are very important in HILIC, influencing the interactions that take place responsible for retention.

5. The best chromatographic separations for the selected analytes were achieved using $HCOONH_4$/HCOOH buffered aqueous mobile phases at high buffering capacity pH and high salt concentrations.

6. No straightforward conclusions were derived about adsorption being the predominant retention mechanism controlling retention of iodinated X-ray media on bare silica and zwitterionic phases, because the results suggested the additional contributions of hydrophilic partition and ionic interactions. Observations also pointed out the presence of hydrogen bonding as relevant interactions. These results confirmed the multimodal retention mechanism characterizing HILIC.

7. A HILIC-HRMS method was successfully developed to determine artificial sweeteners in river and wastewater samples, exhibiting lower matrix effect when compared with other methods.

8. The HILIC-HRMS method developed benefited from the possibility of injecting organic extracts directly into the chromatographic system, involving a significant reduction of the time required for the sample treatment procedure.

9. In the present Thesis the use of mixed-mode sorbents for alternative applications was demonstrated, showing their high potential and versatility for different sample treatment approaches apart from the conventional uses and protocols.

10. For the first time, the selective retention through ionic interactions was proved for a group of benzotriazoles and benzothiazoles which were neutral under SPE conditions. This behavior was promoted by the capability of the analytes to delocalize charge density broadening the potential of mixed-mode ion-exchange sorbents for the extraction of several compounds with particular properties capable of developing stable ion-dipoles.

11. The developed method for benzotriazoles and benzothiazoles based on SPE with mixed-mode sorbents provided cleaner extracts and reduction of matrix effect in the analysis of environmental water samples by LC-HRMS.

12. Successful results were obtained when combining mixed-mode ion-exchange sorbents in pairs with opposite and balanced charge for the simultaneous and selective extraction of basic and acidic pharmaceuticals from river and wastewater samples.

13. When comparing different configurations for combined mixed-mode sorbents, there was no evidence that the weak or strong character of

the functional groups present in the sorbents influenced their performance for the retention of ionic compounds.

14. The excellent capabilities of the combined mixed-mode sorbents to act as simultaneous cation and anion-exchangers, suggested the potential success of polymeric sorbents with zwitterionic properties.

15. The post-polymerization functionalization of polymeric microporous microspheres was a successful route for the preparation of mixed-mode zwitterionic-exchange sorbents.

16. The presence of amphoteric moieties with weak properties within the HPLXPP-WCX/WAX sorbent was confirmed by the selective retention of acidic compounds with strong properties and basic compounds with particular chemical structures. The PP-SCX/WAX and PP-SCX/SAX sorbents exhibited excellent anion-exchange capabilities but absence of cation-exchange properties, an issue that needs to be further investigated.

17. The results obtained in this Thesis are very promising for further research in the development and application of mixed-mode zwitterionic-exchange sorbents for the selective extraction of ionizable compounds with specific structures.

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APPENDIX

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Appendix I. Abbreviations used in this Doctoral Thesis.

4TTR	4-Methyl-1-H-benzotriazole
5TTR	5-Methyl-1-H-benzotriazole
ACE	Acesulfame
ACN	Acetonitrile
AIBN	2,2´-Azobis(isobutyronitrile)
ALI	Alitame
AMPSA	2-Acrylamido-2-methylpropanesulfonic acid
ANTY	Antipyrine
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionisation
ASP	Aspartame
ATE	Atenolol
AU	Adimensional units
BE	Benzoylecgonine
BSA	Benzenesulfonamide
BSAs	Benzenesulfonamide derivates
BT	Benzothiazole
BTR	1-H-Benzotriazole
BTRs	Benzotriazole derivates
BTs	Benzothiazole derivates
C_{18}	Octadecyl
C_8	Octyl
CAFF	Caffeine
CBZ	Carbamazepine
ClBTR	5-Chloro-1-H-benzotriazole
CLO AC	Clofibric acid
COC	Cocaine
COD	Codeine
CYC	Cyclamate
DCE	1,2-Dichloroethane
DEMA	Diethylmethylamine
DFPSE	Dynamic fabric phase sorptive extraction
DICLO	Diclofenac

DMBA	Dimethylbutylamine
DMEA	Dimethylethylamine
dSPE	Dispersive solid-phase extraction
DVB	Divinylbenzene
EDA	Ethylenediamine
EOCs	Emerging organic contaminants
ESI	Electrospray ionization
EtOH	Ethanol
Et-p-BSA	N-ethyl-p-toluenesulfonamide
FEN	Fenoprofen
FPSE	Fabric phase sorptive extraction
FT-IR	Fourier transform infrarred
FWHM	Full width at half maximum
GC	Gas chromatography
GLY	Glycyrrhizic acid
HCD	Higher energy collisional dissociation
HEMA	2-Hydroxyethyl methacrylate
HESI	Heated electrospay ionization
HILIC	Hydrophilic interaction liquid chromatography
HRMS	High resolution mass spectrometry
HXL	Hypercrosslinked
IBP	Ibuprofen
IC	Ion chromatography
ICM	Iodinated X-ray contrast media
ICP	Inductively coupled plasma
ILOD	Instrumental limit of detection
ILOQ	Instrumental limit of quantification
IMSs	Immunosorbents
IPC	Ion pair chromatography
IPR	Ion pairing reagent
IT	Ion trap
k	Retention factor
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection

log D	Partition coefficient
LOQ	Limit of quantification
LSER	Linear solvation energy relationship
MAA	Methacrylic acid
MALDI	Matrix-assisted laser desorption/ionization
MDPV	Methylenedioxypyrvalerone
ME	Matrix effect
MeBT	2-Methylbenzothiazole
MeOH	Methanol
MEPS	Microextraction in packed syringe
Me-p-TSA	*N*-Methyl-*para*-toluenesulfonamide
MeSBT	2-(Methylthio)benzothiazole
MET	Metaprolol
METH	Methadone
MIPs	Molecularly imprinted polymers
MLOD	Method limit of detection
MLOQ	Method limit of quantification
MMC	Mephedrone
MOR	Morphine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NAD	Non-aqueous dispersion polymerisation
NEO	Neotame
NH_2BT	2-Aminobenzothiazole
NHDC	Neohesperidine dihydrochalcone
NMR	Nuclear magnetic resonance
NPLC	Normal phase liquid chromatography
NSAID	Non-steroidal anti-inflammatory drugs
NVIm	N-vinylimidazole
OHBT	2-Hydroxybenzothiazole
o-TSA	*Ortho*-toluenesulfonamide
PCA	Principal component analysis
PETRA	Pentaerythritol triacrylate
PFC	Perfluorinated compounds
PP	Precipitation polimerization

PPCPs	Pharmaceuticals and personal care products
PROP	Propanolol
PS	Polystyrene
PTFE	Polytetrafluoroethylene
p-TSA	*Para*-toluenesulfonamide
PVP	Vinylpyrrolidone
PVP-DVB	Poly(N-vinylpyrrolidonedivinylbenzene
QqQ	Triple quadrupole
QSRR	Quantitative structure–retention relationship
RAMs	Restricted access materials
RAN	Ranitidine
RP	Reversed-phase
RPLC	Reversed-phase liquid chromatography
SAC	Saccharine
SAL AC	Salicylic acid
SAX	Strong anion-exchange
SBSE	Stir bar sorptive extraction
SCX	Strong cation-exchange
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
St-DVB	Styrene-divinylbenzene
STE	Stevioside
STPs	Sewage treatment plants
SUC	Sucralose
TBA	Tetrabutylammonium
TEA	Triethylamine
THF	Tetrahydrofuran
TOF	Time of fligth
TPs	Transformation products
TRI	Trimethoprim
TTR	Tolyltriazole
VBC	Vinylbenzylchloride
WAX	Weak anion-exchange
WCX	Weak cation-exchange

WW	Wastewater
WWTP	Wastewater treatment plant
XTR	5,6-Dimethyl-1H-benzotriazole

Appendix II. List of publications.

Salas D., Borrull F., Fontanals N., Marcé R. M., *Hydrophilic interaction liquid chromatography coupled to mass spectrometry-based detection to determine emerging organic contaminants in environmental samples*, Trends. Anal. Chem. (2017) (to be submitted)(Section 1.1.3.1.).

Salas D., Borrull F., Marcé R. M., Fontanals N., *Study of the retention behavior of iodinated X-ray contrast agents in hydrophilic interaction liquid chromatography, comparing bare silica and zwitterionic stationary phases*, J. Sep. Sci. 37 (2014) 1111–1117 (Section 3.1.1.).

Salas D., Borrull F., Fontanals N., Marcé R. M., *Hydrophilic interaction liquid chromatography and high resolution mass spectrometry as an alternative to determine artificial sweeteners in environmental waters*, Anal. Bioanal. Chem 407 (2015) 4277–4285 (Section 3.1.2.).

Salas D., Borrull F., Marcé R. M., Fontanals N., *Study of the retention of benzotriazoles, benzothiazoles and benzenesulfonamides in mixed-mode solid-phase extraction in environmental samples*, J. Chromatogr. A 1444 (2016) 21–31 (Section 3.2.1.).

Salas D., Borrull F., Fontanals N., Marcé R. M., *Combining cationic and anionic mixed-mode sorbents in a single cartridge to extract basic and acidic pharmaceuticals simultaneously from environmental waters*, J. Chromatogr. A (2017) (submitted)(Section 3.2.2.).

Salas D., Anderson K., Cormack P.A.G., Borrull F., Marcé R. M., Fontanals N., *Synthesis and evaluation of polymeric sorbents with zwitterionic character for the solid-phase extraction of model compounds with acidic and basic properties*, J. Chromatogr. A (2017) (to be submitted)(Section 3.3.1.).

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