PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS

Irene Maijó Ferré

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PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS

Irene Maijó Ferré

DOCTORAL THESIS

Supervised by

Dra. Carme Aguilar Anguera and Dra. Marta Calull Blanch

Departament de Química Analítica i Química Orgànica

UNIVERSITAT ROVIRA I VIRGILI

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2012

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La Dr. CARME AGUILAR ANGUERA, Professora Agregada del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. MARTA CALULL BLANCH, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili.

FEM CONSTAR:

Que la present Tesi Doctoral, que porta per títol: "PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS", presentada per IRENE MAIJÓ FERRÉ per optar al grau de Doctor per la Universitat Rovira i Virgili, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, i que tots els resultats presentats són fruit d'experiències realitzades per l'esmentada doctoranda.

I, per a que consti, expedim aquest certificat a Tarragona, 4 de juny de 2012.

Dra. Carme Aguilar Anguera

Dra. Marta Calull Blanch

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1. INTRODUCTION

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Over the last decade, capillary electrophoresis (CE) has gained growing importance due to the many advantages it offers, including simplicity, high separation efficiency, high resolution, low sample and electrolyte consumption, and short analysis time. However, the concentration sensitivity of CE is generally rather low which limits its applicability when trace analysis is required. The relatively high limits of detection (LODs) in CE are mainly caused by the small sample volume (1-100 nL) that can be loaded onto the capillary due the small dimensions of CE capillaries. Additionally, since the most commonly used detection system is the UV detector, the short optical path length determined by the capillary inner diameter (25-100 μm) contributes to the high detection limits achieved.

Several approaches have been developed to overcome sensitivity issues in CE over the last few decades. With respect to instrumental improvements, more sensitive detectors have been coupled to the CE system, such as electrochemical, chemiluminescence, laser-induced fluorescence (LIF) and mass spectrometry (MS) detectors [1-3]. Despite the high selectivity and the improvement of the sensitivity achieved by these detectors, the reduced dimensions of the separation capillaries remain a limitation for trace analysis. Another approach for improving the sensitivity is the application of preconcentration techniques in CE, which are based on the injection of a large sample volume and its subsequent concentration inside the CE system without degrading the separation. These techniques can be categorized into two groups, depending on the phenomena used to concentrate analytes inside the capillary. The first group of techniques involve the manipulation of the electrophoretic velocity of the analytes. These are known as electrophoretic preconcentration techniques, and include as examples sample stacking, isotachophoresis (ITP), pH-mediated stacking, and sweeping, among others [4-7]. The second group involves the partitioning onto or into a distinct phase, and are known as the chromatographic preconcentration techniques. Among them, solid-phase extraction (SPE) coupled in-line to CE has gained popularity in recent years, as there is no need for modification of the commercial CE instrumentation. This coupling can be extremely efficient and yield high preconcentration factors [8-12].

CE technologies in conjunction with electrophoretic and chromatographic preconcentration techniques have been applied to various fields, such as biological analysis, food analysis, pharmaceutical products, toxicological and forensic analysis, and environmental analysis [5,8,11,13].

Over the last few decades, new chemical compounds have continuously been produced, used and then disposed of into the environment. Different studies that have been carried out in recent years have identified a wide range of organic contaminants, which

have led a growing concern over the environmental and health consequences that they can cause [14-17]. These groups of organic contaminants, known as emerging organic contaminants (EOC), are defined as pollutants that were previously unknown or unrecognized as being of concern, because they have hardly been investigated due to the absence of environmental data and the lack of appropriate analytical methods. These EOCs encompass a diverse group of compounds, including drugs of abuse, pharmaceuticals, personal care products (PCPs), steroids and hormones, surfactants, perfluorinated compounds, flame retardants, industrial additives and agents, and gasoline additives, as well as their transformation products, among others [18-20]. In recent years, great attention has been paid to the presence of these pollutants in the aquatic environment as, in most cases, these compounds correspond to unregulated contaminants which may be candidates for future water-quality regulation depending on their potential health or ecotoxicological effects and on monitoring data regarding their occurrence [19-21].

Among the different EOCs, pharmaceutical and personal care products (PPCPs) are currently the most widely studied compounds [22-25]. In general, these compounds are introduced directly into the environment through recreational waters and domestic, urban and industrial wastewaters discharges, waste disposals, or released from products or materials that contain them. Taking their physico-chemical properties into account, some groups of PPCPs (e.g. parabens) are effectively removed in the wastewater treatment plants (WWTP). However, these compounds are considered to be pseudo-persistent, since their continuous discharge into the aquatic environment compensates for the rapid removal and/or transformation thereof. They may therefore cause negative effects in the short or long term in the environment [22]. Other groups of PPCPs (e.g. non-steroidal anti-inflammatory drugs (NSAIDs) and UV-filters) are not completely eliminated in WWTPs and may therefore be present at levels up to micrograms per litre, in river streams, lakes and even in groundwater [23,26-29].

It has now been demonstrated that some of these compounds can act as endocrine-disrupting chemicals, exhibiting weak or moderate estrogenic and other hormone-related activity. Although toxicity is relatively low in most cases of these compounds, they can be precursors of other chemicals considered to be priority organic pollutants [15,30-34]. The continuous release of these kinds of apparently harmless chemicals into the environment highlights the need to develop suitable analytical methodologies for the monitoring of such substances and their degradation products in different environmental compartments.

Liquid chromatography (LC) and gas chromatography (GC) continue to be the predominant techniques for the determination of organic pollutants, their metabolites and transformation products in environmental samples [22,23,25]. Recent advances in CE, such as the possibility of using more sensitive detectors and preconcentration techniques, in addition to its many other advantages, have made this technique more competitive in the analysis of environmental samples [13,24].

In light of the facts mentioned above, the first part of the introduction of this Doctoral Thesis refers to pharmaceuticals and personal care products, describing in detail the groups of compounds that are studied, which include NSAIDs, parabens and UV-filters. Their main characteristics, some examples of the results of research regarding their presence in various types of water samples and an overview of the use of CE for their determination have been included. In the second part of the introduction, a detailed description is provided of the electrophoretic and chromatographic preconcentration techniques developed for the preconcentration of the studied PPCPs. After the introduction, the main objectives of this Doctoral Thesis are set out. The third chapter presents the results and discussion of the studies derived from the experimental research included in paper format. Finally, the main conclusions that can be drawn from the studies are presented.

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1.1. Pharmaceutical and personal care products

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Pharmaceutical and personal care products constitute one of the most commonly detected groups of EOCs in the aquatic environment, due to either their recalcitrant character, polarity and/or continuous input into the environment that promotes their spread along watercourses [22-25]. Pharmaceuticals are substances used in the diagnosis, treatment, alteration or prevention of abnormal health or structural/function conditions in the body. It is estimated that approximately 3,000 different substances are used as pharmaceutical compounds, including analgesics, NSAIDs, lipid regulators, β-blockers, antibiotics and so on [20]. The active ingredients of PCPs are a broad range of organic compounds currently used as additives in different products widely used in daily life, such as soaps, lotions, gels, cosmetics, household and foodstuffs, among others. There are different groups of PCPs, including synthetic musk fragrances, antimicrobials, sunscreen agents, insect repellents and preservatives, among others.

In the case of pharmaceuticals, one of the main routes of entry into the environment is from treated patients, and in this case the pharmaceutical may enter as a parent compound or as a metabolite. The other main route is via direct release into the wastewater system from manufacturing, hospitals or domestic discharges. In contrast, PCPs can reach surface waters (river, lakes, coastal sea water) via release from the skin during swimming and bathing or through wastewater systems [23,28,29].

PPCPs can be found at very low concentrations in environmental waters. Consequently, there is a growing need to develop reliable analytical methods which enable a rapid, sensitive and selective determination of these PPCPs in environmental water samples. Efficient separation techniques that rely on sensitive and selective detection systems are required. Furthermore, the low concentrations found in complex matrices require an extraction and/or preconcentration step, and in some cases a clean-up step before the separation technique [22-25].

Different extraction techniques have been used to clean up the sample and preconcentrate analytes of interest. The most widely used technique for the determination of PPCPs in water matrices has been SPE, which has the advantage of using a wide variety of potential sorbents [26,35-58]. Another current trend in the development of extraction techniques involves the concept of ‘green chemistry’, which is concerned with reducing the large amounts of solvents used in conventional extraction techniques. Some of the techniques that have been developed to this end include solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), liquid-phase microextraction (LPME), and liquid-liquid microextraction (LLME) [59-75].

Not only efficient extraction techniques, but also efficient and reliable separation methods are required for determination of the occurrence and fate of PPCPs at environmental levels. Gas chromatography-mass spectrometry (GC-MS or GC-MS-MS) methods [35-48,59-72] have greatly contributed to the characterization of small apolar contaminants in water whereas liquid chromatography-mass spectrometry (LC-MS or LC MS-MS) based methods [26,49-58,73-75] have been utilized more recently to extend the investigation of water contaminants to non-volatile, (highly) polar and thermally labile compounds. Capillary electrophoresis, in combination with a preconcentration technique and/or a sensitive detector, has also been used in the determination of PPCPs [13,24,76].

The studies presented in the experimental part of this Doctoral Thesis have focused on the study of different preconcentration techniques to decrease the LODs of CE for the determination of a group of PPCPs, with the main aim being the ability to apply the developed methodology for the analysis of environmental waters. The studied contaminants included non-steroidal anti-inflammatory drugs, which are one of the most commonly used agents in both human and veterinary use, and personal care products, in particular parabens and UV-filters, which are used in several everyday consumer products.

1.1. 1. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs are analgesics and the most commonly used anti-inflammatory pharmaceuticals worldwide nowadays. They are mainly used to treat pain, inflammation and fever in animal and human species. These pharmaceutical compounds are weak acidic compounds, due to the carboxylic groups or to keto-enol tautomerism, with pK_a values between 3 and 7, polar, and highly soluble in aqueous media. The structure of some of the most commonly used NSAIDs is shown in Figure 1.

As discussed above, pharmaceuticals are not completely removed in WWTPs and some of them can enter the environment, either unaltered, in their metabolite form or as transformation products. Studies conducted in various countries around the world have shown that NSAIDs are the most frequently found pharmaceuticals in wastewaters at low micrograms per litre, as many of them are available in some countries without the need for a prescription [26,36,52]. Pedrouzo *et al.* [26] found ibuprofen with a maximum concentration of 4,215 ng mL^{-1} in influent water samples from WWTPs. However, these high concentrations decreased to a maximum of 955 ng mL^{-1} in the

effluent water samples. Moreover, NSAIDs have also been found in river water samples at concentration levels ranging between 3.7 ng mL^{-1} and 50 ng mL^{-1} for ibuprofen, 4.7 and 62 ng mL^{-1} for naproxen, and 24 ng mL^{-1} and 931 ng mL^{-1} for diclofenac [37].

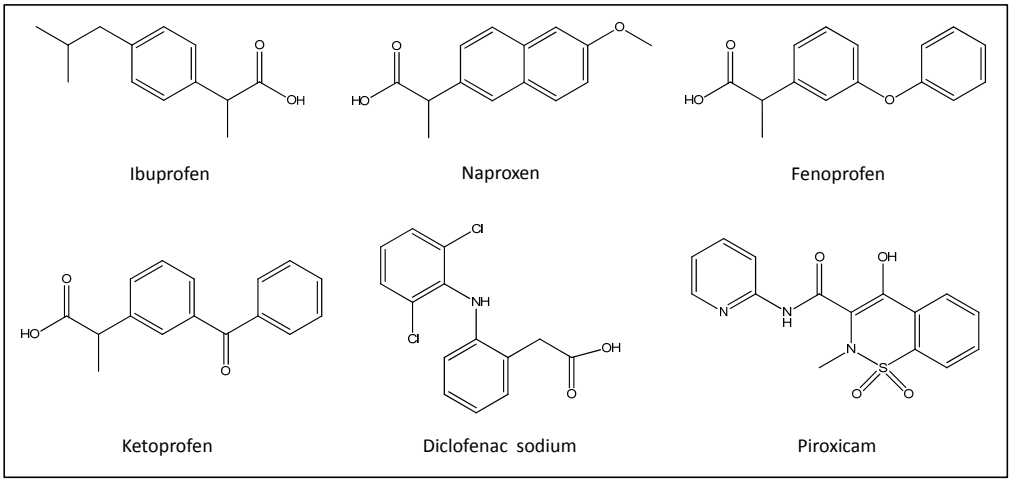

Figure 1. Chemical structures of the most commonly used NSAIDs.

At present, the most frequently used techniques for the determination of different pharmaceutical compounds, including NSAIDs, in environmental waters are GC and LC coupled to MS or MS-MS [22-25]. Furthermore, a variety of techniques are used to extract pharmaceutical compounds from environmental waters, although the simultaneous extraction of all target analytes in one single SPE step is the most widely employed approach [26,35-42,49-56].

Since the early nineties, the use of CE in the analysis of NSAIDs has increased, mainly due to recent advances in instrumentation and the technique's high versatility. Macià *et al*. [76] presented a review with a complete overview of the different approaches that had appeared in the literature before 2008 regarding the use of various CE modes for the analysis of NSAIDs. In recent years, different papers have been published in the literature about the determination of NSAIDs by CE in different samples, mainly water and biological samples. As a result, it can be highlighted that, apart from the off-line preconcentration and/or clean-up step which are normally involved in these methods, the application of CE for the analysis of these kind of samples generally requires a chromatographic or electrophoretic preconcentration step as well, in order to achieve the concentration levels at which these drugs are usually found [77-90].

Capillary zone electrophoresis (CZE) is the most widely used CE separation mode for NSAIDs analysis due to its great simplicity. The current trend when using this separation

mode is the use of different preconcentration mechanisms either electrophoretic or chromatographic, and also the use of more sensitive detectors such as MS. These strategies have mainly been applied for the analysis of different biological samples. For instance, Botello *et al.* [77] applied pseudo transient isotachophoresis (ptITP) for the determination of NSAIDs in biological samples, specifically human plasma and urine. A simple liquid-liquid extraction (LLE) was carried out as a sample pretreatment, achieving LODs ranging from 160 ng mL^{-1} to 260 ng mL^{-1} and 320 ng mL^{-1} to 750 ng mL^{-1} for plasma and urine, respectively. Dawod *et al*. [78] developed an electrokinetic supercharging (EKS) preconcentration method for the analysis of seven NSAIDs in wastewater. EKS is the combination of an electrokinetic injection of the sample under field-amplified conditions (field-amplified sample injection, FASI) and transient isotachophoresis (tITP). Under the optimal conditions, 2,400-fold improvement in detection sensitivity was achieved with LODs ranging from 50 pg mL^{-1} to 180 pg mL^{-1}. To demonstrate the potential of the developed EKS method, wastewater was injected directly, without any pretreatment step, and the LODs were approximately 10 times higher than when compared to those obtained using standards. Botello *et al*. [79] also explored the applicability of the EKS preconcentration technique to determine NSAIDs in different sample matrices, in particular environmental and biological samples. The strategy enhanced detection sensitivity up to 2,000-fold in standard samples, and LODs ranging from 0.9 ng mL^{-1} to 1.1 ng mL^{-1} and 2 ng mL^{-1} to 9 ng mL^{-1} were achieved for river water samples and human plasma samples, respectively. Dawod *et al.* [80] developed a valuable modification for the EKS system, namely counter-flow EKS (CF-EKS) and applied it for the separation and on-line preconcentration of seven NSAIDs in water samples. In CF-EKS, a hydrodynamic counter-flow is applied during the electrokinetic injection of the analytes within the EKS system. This counter-flow minimizes the introduction of the sample matrix into the capillary, and this allows longer injections of the sample. However, the way in which the commercial instrument applies the counter-pressure does not allow the coupling of CE to MS. Under the optimal conditions, CF-EKS allowed an enhancement in detection sensitivity of 11,800-fold, and LODs from 10.7 pg mL^{-1} to 47.0 pg mL^{-1} in standards. The developed method was validated and then applied to the determination of the studied NSAIDs in drinking water as well as wastewater samples from Hobart city. Recently, the same research group combined EKS with a positive pressure during injection (PA-EKS) to improve the preconcentration, and at the same time developed a system that has the potential to be MS compatible [81]. Under these conditions, detection limits for seven NSAIDs were in the range of 6.7 pg mL^{-1} to 18.7 pg mL^{-1} and an enhancement in detection sensitivity of almost 50,000-fold was obtained.

Another trend when using CZE is the combination with SPE. In one such case, Macià *et al.* [82] investigated the use of a SPE coupled in-line to CE for the preconcentration of naproxen, using the reverse-phase sorbent C18. The detection sensitivity was enhanced 1,820-fold and a LOD of 0.2 ng mL^{-1} was achieved in standards. Tap water samples were analysed by off-line SPE followed by in-line SPE-CE achieving in this case a LOD of 10 ng mL^{-1}. More recently, Navarro *et al.* [83] described a CZE determination of several NSAIDs in urban wastewaters using a hollow fibre membrane liquid-phase microextraction, providing LODs in the range of 0.25 ng mL^{-1} to 0.86 ng mL^{-1} in standards. The method was applied to the determination of the seven anti-inflammatories in wastewaters, and five of them were detected in some of the analysed samples. Naproxen and salicylic acid could be quantified with concentrations ranging from 1.43 ng mL^{-1} to 2.12 ng mL^{-1} for naproxen and from 1.98 ng mL^{-1} to 2.87 ng mL^{-1} for salicylic acid.

Capillary electrochromatography (CEC) is a hybrid separation technique which combines the features of LC and CE, and has gained much attention in recent years. Recently, Hsu *et al.* [84] developed a poly(stearyl methacrylate-divinylbenzene) monolithic column, which was prepared by a simple *in situ* polymerization for the CEC separation of a group of nine NSAIDs. Furthermore, a field-amplified sample injection pre-concentration was applied to CEC coupled with MS (TOF-MS) for the determination of the NSAIDs in water samples. The method provided LODs in the range of 0.01 ng mL^{-1} to 0.19 ng mL^{-1} for standard samples.

In the literature, different micellar electrokinetic capillary chromatography (MEKC) and microemulsion electrokinetic capillary chromatography (MEEKC) methodologies have also been reported for the determination of NSAIDs in different samples. These separation modes have been combined with electrophoretic preconcentration techniques, mainly with sample stacking techniques. For example, Macià *et al.* [85] examined stacking with reversed migration micelles (SRMM), stacking with reverse migrating micelles-anion selective exhaustive injection (SRMM-ASEI) and field-enhanced sample injection with reverse migration micelles (FESI-RMM) for the determination of some NSAIDs after an off-line SPE sample pretreatment in mineral waters. The detection sensitivity of the stacking approaches was enhanced up to 143-, 250-, and 60-fold, respectively. Almeda *et al*. [86] applied a combination of an off-line SPE and large volume sample stacking (LVSS) with polarity switching in MEKC for the determination of traces of NSAIDs in saliva. The developed SPE-LVSS-MEKC method provided 500-fold improvement in detection sensitivity. Macià *et al*. [87] also investigated stacking with a reverse migrating pseudostationary phase (SRMP) in MEEKC for analysis of tap water

samples. When the method was combined with an off-line SPE, the LODs achieved for spiked tap water samples were in the 0.1 ng mL^{-1} to 0.23 ng mL^{-1} range. More recently, Kuo *et al.* [88] used MEEKC in combination with field-amplified sample injection to determine NSAIDs in several water samples (river and groundwater). The method provided between 1,400-fold and 6,100-fold improvements in the detection limits, and LODs were in the range of 0.03 ng mL^{-1} to 0.3 ng mL^{-1} for standard samples. The compounds were determined in river and groundwater samples after a SPE treatment followed by FASI-MEEKC method, their concentration in the river and groundwater samples were 223 ng mL^{-1} and 60 ng mL^{-1}, respectively.

Recently, Quirino *et al.* [90] described a two-step stacking of hypolipidaemic drugs, NSAIDs and herbicides by combining two different electrophoretic preconcentration strategies, sweeping and micelle to solvent stacking, using cationic cetyl trimethyl ammonium chloride (CTAC) micelles in co-electroosmotic flow capillary zone electrophoresis. An 18- to 21-fold increase in peak height sensitivity was obtained for the NSAIDs and the LODs ranged from 0.05 mg L^{-1} to 0.55 mg L^{-1} in standards.

1.1.2. Parabens

Parabens (p-hydroxybenzoic esters) are the most common preservatives and antimicrobial agents used in personal care, pharmaceutical and food products. The widespread use of parabens as preservatives comes from their high antimicrobial activity, inertness, worldwide regularity acceptance, biodegradability, stability to pH (effective between pH 4.5 and 7.5), temperature, and low cost [91]. The most widely used parabens are methyl and propyl paraben, because of the synergistic effect when using both compounds together [92]. Other parabens include ethyl, isopropyl, butyl, isobutyl and benzyl parabens. Figure 2 shows the general structure of parabens.

The European Economic Community (EEC) Directives 76/768/EEC and 95/17/EC permit the use of parabens in cosmetics with a maximum concentration of 0.4% w/w for each paraben and a total maximum concentration of 0.8% w/w, expressed as p-hydroxybenzoic acid (PHBA) [93]. The USA Food and Drug Administration regulations limit paraben concentration in food to 0.1% w/w [94] and, even though they are not regulated in pharmaceuticals, levels do not exceed 1% w/w [91,95]. Therefore, the significance of developing methods for the assay of these substances in cosmetic, food and pharmaceutical products with high precision and accuracy is needed. With this in mind, the use of GC and LC coupled to MS or MS-MS [91,95-102] can be highlighted.

However, in recent years, CE has become a more popular separation technique for the determination of this kind of analytes [103-113]. Different CE modes, such as CZE [103-105], MEKC [106-109], MEEKC [106,110], and CEC [111-113], have recently been used for that purpose. Generally, the detection system used is a UV detector, although there are some studies with amperometric detection (AD), which affords high sensitivity and good selectivity for electroactive species [103,109].

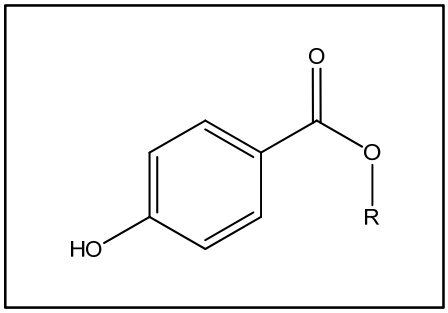

Figure 2. General structure of parabens.

As mentioned earlier, parabens are widely used as preservatives in cosmetics, pharmaceuticals and food products. Therefore, these compounds are continuously released through urban and industrial wastewater, which is their main entry route into the environment. Parabens are considered as 'pseudo-persistent' compounds and so they can be detected in environmental water samples at low concentration levels [32,44-46,114]. Their determination is normally achieved with a sample preconcentration, generally based on SPE followed by GC or LC, both combined with MS or MS-MS [43-46,59-62]. For instance, González-Mariño *et al.* [46] found methylparaben and n-propylparaben in influents waters of a WWTP in the surroundings of Santiago de Compostela (Spain) at a concentration of up to 5.1 ng mL^{-1} and 1.1 ng mL^{-1}, respectively, whereas the concentrations for the effluent waters were under the limit of quantification. Parabens have also been detected in river water samples at concentrations of up to 48 pg mL^{-1} for methylparaben and 8 pg mL^{-1} for ethylparaben [44].

Determination of parabens by CE in environmental samples has not been widely studied. This kind of sample requires a preconcentration technique in order to achieve the low LODs at which these compounds can be found. At the time of writing this Doctoral Thesis, only two works based on electrophoretic preconcentration techniques in CE have been described to determine parabens in environmental waters. Blanco *et al.* [115,116] carried out a preconcentration step by off-line SPE followed by a large volume sample stacking preconcentration technique combined with nonaqueous capillary

electrophoresis (NACE) for the quantification of parabens in wastewater samples. Concentrations of up to 8.4 ng mL^{-1} were found in influents waters of WWTP, with the highest levels corresponding to methylparaben and propylparaben.

1.1.3. UV-filters

Organic UV-filters are very often found in beauty creams, shampoos and other personal care products to protect the skin from UV radiation. They are increasingly being used as a result of growing concern about UV radiation and skin cancer. Most of these compounds are lipophilic compounds (log k_{ow} 4-8) with conjugated aromatic rings and are relatively stable with respect to biotic degradation. Based on research into the use and the effects of old and new formulations and their toxicity, the list of compounds permitted by legislation is regularly updated. The EU Cosmetics Directive permits the commercial use of 27 organic UV-filters, including benzophenones, p-aminobenzoic acid and derivates, salicylates, cinnamates, triazines, benzimidazole derivates and dibenzoylmethane derivates s, as well as compounds like octocrylene and benzylidene malonate polysiloxane [117]. Figure 3 shows the structure of the four UV-filters studied in the present Doctoral Thesis.

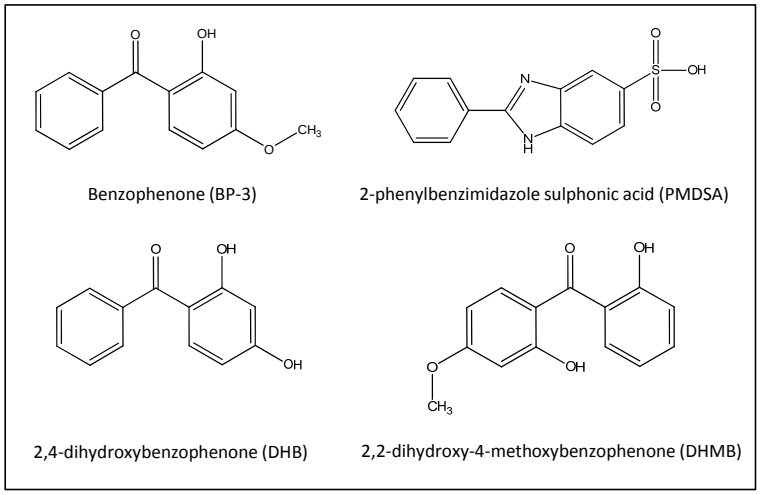

Figure 3. Structure of the four UV-filters studied in the present Doctoral Thesis.

There are three main regulatory bodies that control the amount and types of UV-filters used in sunscreen and cosmetic products, namely the EU Cosmetics Directive, the US Food and Drug Administration (FDA) and the legislative bodies in Japan [118-120]. Therefore, in the same way as with the paraben compounds, sensitive analytical

methods are needed in order to determine the presence of UV-filters in sunscreen and cosmetic products. LC combined with MS is the technique that is usually used for the determination of these compounds in these kinds of samples [121-124], although CE has also been applied for the determination of UV-filters compounds [125-129].

UV-filters reach surface waters (river, lakes, coastal sea water) via release from the skin during swimming and bathing or through wastewater systems. Most UV-filters are highly lipophilic and not very degradable in sewage treatment plants and are therefore bound to accumulate in the environment [23,26-29]. Available procedures for the analysis of UV-filters in water samples are mainly based on a previous preconcentration technique, such us SPE, LLE, SBSE and SPME, and their determination by GC or LC coupled with MS or MS-MS detection (MS) [47-48,57-58,63-71,74-75]. For instance, BP-3 has been detected in river water samples at concentrations between 6 pg mL^{-1} and 28 pg mL^{-1} [74]. Negreira *et al.* [58] found three common UV-filters, BP-1, BP-3 and BP-4 in influents waters of a WWTP with concentrations ranging from 30 pg mL^{-1} for BP-1 up to 1,600 pg mL^{-1} for BP-4. Concentrations of BP-1 and BP-3 were considerably reduced in the effluent waters of the plant. However, its efficiency was poor for BP-4. A very recent work of Nguyen *et al*. [70] focuses on the determination of different UV-filters (BP-3 and PBSA) in seawater collected around seaside resorts of Liguria and values for BP-3 ranging from 33 pg mL^{-1} to 118 pg mL^{-1} were reported.

At the time of writing this Doctoral Thesis and to the best of our knowledge, there has only been one study which investigates the determination of UV-filters compounds in environmental water samples by CE. Den *et al.* [130] developed a method based on dispersive liquid-liquid microextraction and MEKC determination of 2,4-dihydroxy-benzophenone (HBP) and 2-hydroxy-4-methoxy-benzophenone (HMBP) in environmental water samples after topical skin application. Under the optimal conditions, sensitivity enhancements of 209-fold for HBP and 195-fold for HMBP were obtained. The detection limits were 0.52 mmol L^{-1} for HBP and 0.29 mmol L^{-1} for HMBP in standard samples.

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1.2. Sample preconcentration techniques in capillary electrophoresis

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As mentioned earlier, one of the main drawbacks of CE is its low sensitivity. This lack of sensitivity is associated with both the short optical path-length of the capillary used as a detection cell (for spectrophotometric detection) and the small sample volumes that are usually injected. The use of a more sensitive detector, such as MS or LIF, increases the sensitivity of the technique [1-3], but the main approach used in order to improve the sensitivity of CE is increasing the volume of sample introduced into the capillary, which allows the amount of analytes in the CE system to be increased. There are different strategies for achieving this increase in the amount of analytes injected and subsequent concentration, which can be categorized into two groups, namely electrophoretic preconcentration techniques and chromatographic preconcentration techniques [4-12].

The electrophoretic preconcentration techniques are the most frequently used approaches for improving sensitivity in CE [4-7]. The principle of these techniques relies on differences in migration velovity of the analytes in different zones, causing them to be focused or preconcentrated. The differences in migration velocity are caused by the change of different properties between the sample and the background electrolyte (BGE) zone, such as conductivity (ionic strength), additive concentration (analyte-additive-interactions) and buffer pH. Depending on the distinct focusing mechanism used, four major preconcentration techniques have been reported in CE: sample stacking, transient isotachophoresis, sweeping and dynamic pH junction [4-7].

Chromatographic preconcentration techniques can be very efficient yielding high concentration factors. In this approach, analytes from a large volume of sample are concentrated on a sorbent and then eluted in a small solvent volume, leading to lower detection limits. Solid-phase extraction and solid-phase microextraction are the most popular among the different chromatographic-based techniques [8-12]. The simplest way to combine SPE and CE is to perform the two steps separately, i.e. in the off-line mode. Off-line SPE-CE has frequently been used over the past two decades for many applications. As this approach is rather laborious and time-consuming, there has been a trend towards automation in recent years. As a result, over the last few years, many studies have focused on developing in-line, at-line and on-line combinations of SPE and CE [8-12].

In the following sections, there will be a discussion of the electrophoretic and chromatographic preconcentration techniques used in combination with CE to determine the compounds studied in this Doctoral Thesis. In particular, the preconcentration techniques studied are sample stacking, sweeping and in-line SPE-CE.

1.2.1. Sample stacking techniques

Sample stacking strategies are used for the preconcentration of analytes in diluted samples. During the stacking process, analytes present at low concentration levels in a long injected sample zone are concentrated in a short zone (stack) for their subsequent separation and detection. The preconcentration mechanism is based on a conductivity difference between the sample zone (low conductivity zone) and the BGE zone (high conductivity zone) and consequently, on the change of the electrophoretic velocities of the analytes between these two zones.

In the literature, stacking techniques have been reviewed by a number of authors over the past decade [4-7], with respect to various procedures that share the same principles, although referred to in a number of ways. In this Doctoral Thesis, the sample stacking techniques have been classified depending on whether the injection mode applied is hydrodynamic or electrokinetic, and with respect to whether the sample matrix is removed before the separation or not. Furthermore, in the case of sample matrix removal, two subgroups can be described, depending on whether the sample matrix is removed with or without polarity switching. Moreover, it should also be noted that the stacking techniques can be applied in all the CE separation modes. Table 1 presents the sample stacking techniques most commonly used to decrease the LODs in CE.

Table 1. Sample stacking preconcentration techniques in CE.

	CZE	**MEKC**	**MEEKC**
Without sample matrix removal			
Hydrodynamic injection	FASS or NSM	FASS or NSM	FASS or NSM
Electrokinetic injection	FASI	FASI	FASI
With sample matrix removal			
		With polarity switching	
Hydrodynamic injection	LVSS	REPSM	REPSM
Electrokinetic injection	FESI	FESI	FESI
		Without polarity switching	
Hydrodynamic injection	LVSEP	SRMM	SRMP
Electrokinetic injection	FAEP	FESI-RMM	FESI-RMP

The first approaches of sample stacking, namely *Field-Amplified Sample Stacking* (FASS) or *Normal Stacking Mode* (NSM), were carried out without removing the sample matrix and the injection was performed hydrodynamically [4]. FASS, which was first demonstrated by Mikkers *et al.* [131], involves the hydrodynamic injection of a long plug of sample, prepared in a low conductivity matrix, into a capillary previously filled with a high-conductivity BGE in order to establish a discrete electrolyte system. The electric field in the sample zone is much higher than that in the BGE. Under these conditions, the application of a separation voltage across the capillary causes ions in the sample matrix to migrate faster than in the separation medium, so they slown down when they enter the BGE. This leads to the formation of a narrow zone of analytes at the boundary of the sample zone and the BGE zone. FASS can be used to preconcentrate analytes with a lower mobility than the electroosmotic flow (EOF), as the separation is performed when the EOF mobility is greater than the electrophoretic mobility of the analytes, and anionic and cationic analytes can be determined at the same time.

One of the major restrictions of FASS is the limited sample volume that can be injected. Chien and Burgi [132] calculated that the maximum volume of the capillary that can be filled is typically only about 5% of the total capillary volume, because larger volumes induce broadening due to electroosmotically induced pressure waves arising from the different magnitude of the EOF in the BGE and sample zones. Furthermore, if too large a volume of sample is injected, an insufficient length of capillary is left for the separation of the analytes. Due to this limitation in the sample volume, this preconcentration technique does not allow high sensitivity enhancement factors to be obtained, and generally detection sensitivity improvements between 15- and 50-fold have been achieved [133].

As mentioned above, the sample stacking techniques can also be performed by an electrokinetic injection of the sample. As is well known, this kind of injection allows a higher amount of analytes to be introduced into the capillary than the hydrodynamic injection, especially when analytes have high electrophoretic mobility. However, the electrokinetic injection has some limitations, as this injection is less precise, is highly dependent on the sample matrix, and analytes are discriminated on the basis of their electrophoretic mobility.

Field-Amplified Sample Injection (FASI) works on a similar principle to FASS. The difference between the two techniques is the injection mode used. In FASI, the sample is injected electrokinetically. It was noticed that introducing a short plug of a low-conductivity solution (solvent plug) before the electrokinetic injection of the sample

provided a high electric field strength from the beginning of the injection, which facilitated the entrance of the analytes into the capillary [132]. The solvents that are more widely used as solvent plugs include water and mixtures of water with methanol or acetonitrile [134-137]. Macià *et al*. [134] demonstrated that the low conductivity of the solvent plug is a key parameter if optimal sensitivity is desired. Wu *et al*. [135] indicated that higher reproducibility could be obtained if a water plug was used. When FASI is applied, anions and cations cannot be preconcentrated simultaneously, due to the electrokinetic injection of the sample which is selective for both kinds of ions. FASI can provide higher preconcentration factors than FASS and the detection sensitivity can be improved by to a factor of 500-fold. Even though this approach is exceptionally simple, FASI suffers from the same shortcomings as FASS in relation to the injection length of the sample [133].

Due to the simplicity of the mentioned stacking techniques, there are a great number of applications reported in the literature which use them as preconcentration strategies. Among the different compounds determined by these preconcentration techniques in combination with CE, examples worth highlighting include tetracyclines in biological fluids [138,139], diuretics in urine [140], pesticides in foods [141], allantoin/uric acid ratio and malondialdehyde in human plasma [142,143], haloacetic acids in drinking water [144], adenine nucleotides [145], fluoroquinolone antibiotics in chicken [146], urinary neurotransmitters [147] and biomarkers [148]. For instance, Zhou *et al.* [148] applied FASI using a fused silica capillary coated with gold nanoparticles embedded in poly(diallyldimethylammonium) chloride for the determination of indoxyl sulphate, homovanillic acid, and vanillylmandelic acid. The LODs for the three analytes in standard samples by using the stacking preconcentration and an electrochemical detection were about 75 nM, which were significantly below their normal physiological levels in biological fluids. The developed methodology was applied to the direct analysis of these analytes and other interfering chemicals including uric and ascorbic acids in urine samples without any off-line sample treatment or preconcentration. He *et al.* [146] developed simultaneous determination of nine fluoroquinolone antibiotics (FQs) by FASS-CE-UV with a previous off-line poly(methacrylic acid-co-ethylene glycol dimethacrylate) monolith microextraction (PMME). The proposed PMME–FASS–CE method was applied to the determination of FQs residues in chicken samples, and LODs were found to be in the range of 2.4 ng g^{-1} to 34.0 ng g^{-1}.

A number of ingenious approaches have been developed to overcome the shortcomings of FASS and FASI and provide a greater increase of sensitivity. In the 1990s, Chieng and Burgi [132] examined the ways in which capillaries could be loaded with more than 5%

of their volume without detracting from efficiency. Realizing that the loss in efficiency arose from the presence of the sample matrix in the capillary and also from the EOF mismatch with the BGE, they devised a methodology by which the analytes would remain stacked while the sample matrix was removed. The methodology was named *Large Volume Sample Stacking* (LVSS). This approach was performed by using a similar buffer system as in the case of FASS, but with the main difference being switching the electrode polarity to reverse the EOF and remove the sample matrix, while the stacked analytes move back towards the detector.

The polarity switching is a critical step for the stacking preconcentration, as it has to be reversed before the stacked analyte zone exits the inlet of the capillary. To switch the polarity, the current must be monitored and the voltage should be changed when the current reaches a predefined proportion of the current value when the capillary is completely filled with BGE. This is normally set between 95% and 99% of the total current.

A schematic representation of LVSS applied in CZE to preconcentrate anionic analytes is shown in Figure 4. In the initial step, the sample is dissolved in either low conductivity buffer or water. After the conditioning of the capillary with a high-conductivity BGE, a large volume of sample is then injected hydrodynamically into the capillary (a). Subsequently, the BGE is placed in the inlet vial and a negative voltage (stacking voltage) is applied in order to reverse the direction of the EOF (b). Thus, the EOF pushes the sample matrix towards the inlet while the anionic analytes move quickly toward the outlet and they are accumulated or stacked at the interface between the sample matrix and the BGE (c). The current of the system increases in absolute terms as the capillary is filled with the BGE that comes from the outlet and the sample matrix is removed through the inlet. When the current reaches 95% to 99 % of the current value that exists when the capillary is totally filled with the BGE, the polarity is switched (d). With a positive voltage, the electrophoretic separation begins. The analytes migrate toward the detector due to the EOF mobility, since this magnitude is greater than the effective electrophoretic mobility of anions (μ_{e1} and μ_{e2}) (e).

LVSS is an operationally demanding procedure. Thus, obtaining reproducible results requires careful monitoring of the current by the analyst. In addition, as with the previous techniques (FASS and FASI), the samples must have a relatively low conductivity and the technique is limited to analytes with mobility that is lower than the EOF. Moreover, when LVSS is applied, anions and cations cannot be preconcentrated simultaneously.

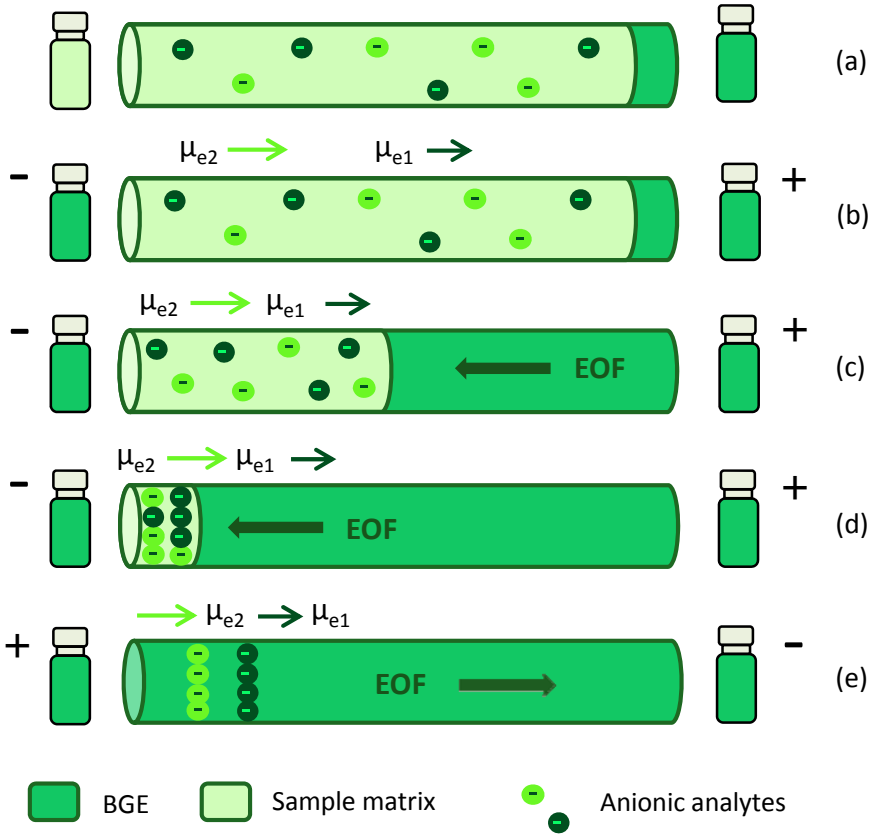

Figure 4. Schematic representation of *Large Volume Sample Stacking* (LVSS) in CZE for the analysis of anions.

Since its introduction, LVSS has also been applied in MEKC and MEEKC modes, but in those cases, this preconcentration technique has been referred to as *Reversed Electrode Polarity Stacking Mode* (REPSM). In general, with the use of LVSS and REPSM, enhancement factors ranging from 20-fold to 500-fold can be achieved. Some examples of using these techniques are the determination of haloacetic acids in drinking waters [144], NSAIDs in saliva [87], peptides in human saliva and cerebrospinal fluid (CSF) [149], speciation of organomercury in fish samples and human hair samples [150], degradation products of metribuzin in soil samples [151], β-lactam antibiotics in water samples and milk samples [152,153], cephalosporins [154], gold nanoparticles (Au NPs) [155], melamine and related triazine in flour products [156], and pesticides in red wine samples [157], among others.

Burgi [158] introduced a method in which polarity switching was not necessary to remove the sample matrix. This was achieved by changing the EOF direction during the

stacking/matrix removal via a dynamic EOF reversal agent introduced in the BGE. This method was referred to as *Large-Volume Stacking using the EOF Pump* (LVSEP) or LVSS using an EOF pump. For example, Burgi [158] used diethylenetriamine as an EOF reversal agent in the BGE. Apart from this agent [158,159], different reversal agents have been evaluated such as cationic surfactants as cetyltrimethylammonium bromide (CTAB) and tetradecyltrimethylammonium bromide (TTAB) [160-162], as well as modifiers of the capillary surface, such as poly(ethylene oxide) and poly(vinyl alcohol) [163-166]. Another strategy for removing the sample matrix without polarity switching is through the suppression of the EOF. For that purpose, some authors have used a NACE mode [115,134,167,168] or a low pH BGE [86,88,169-171], which is the most widely used approach. In the case of suppressing the EOF, the technique is limited to analytes with high mobilities.

LVSEP has also been used in combination with MEKC and MEEKC and, in this case, the techniques are referred to as *Stacking with Reverse Migrating Micelles* (SRMM) and *Stacking with Reverse Migrating Pseudostationary Phase* (SRMP), respectively.

Some applications of these techniques to increase sensitivity include the determination of herbicides in drinking water [160,167], parabens in river water samples [115], methotreate and its metabolites in human plasma [165], NSAIDs in environmental water samples [88], illegal drugs in urine samples [169], ddATP metabolite [159], peptides [164], and Fe complexes [161].

As mentioned previously and as can be observed in Table 1, the two developed preconcentration strategies involving a sample matrix removal, LVSS and LVSEP, can also be performed by an electrokinetic injection of the sample. In 1999, Quirino and Terabe [172] developed a FASI methodology involving matrix removal by effect of a polarity switching and they named this new approach *Field-Enhanced Sample Injection* (FESI). This methodology combines the removal of the sample matrix during injection, as in the case of LVSS, with the high preconcentration power of an electrokinetic injection. It is important to highlight that sensitivity enhancement factors of around 1,000-fold have been reported with this technique [173,174]. FESI has the same operational requirements as LVSS, namely the need for a careful monitoring of the current, the samples have to be prepared in a low conductivity matrix, no possibility of analysing anions and cations simultaneously, and the limitation for analytes with low mobility. A schematic representation of FESI applied in CZE is shown in Figure 5. A model to preconcentrate anionic analytes has also been used. After the conditioning of the capillary with the BGE, a solvent plug is injected hydrodynamically into the capillary (a). The capillary inlet is then inserted into the sample solution and a negative voltage is

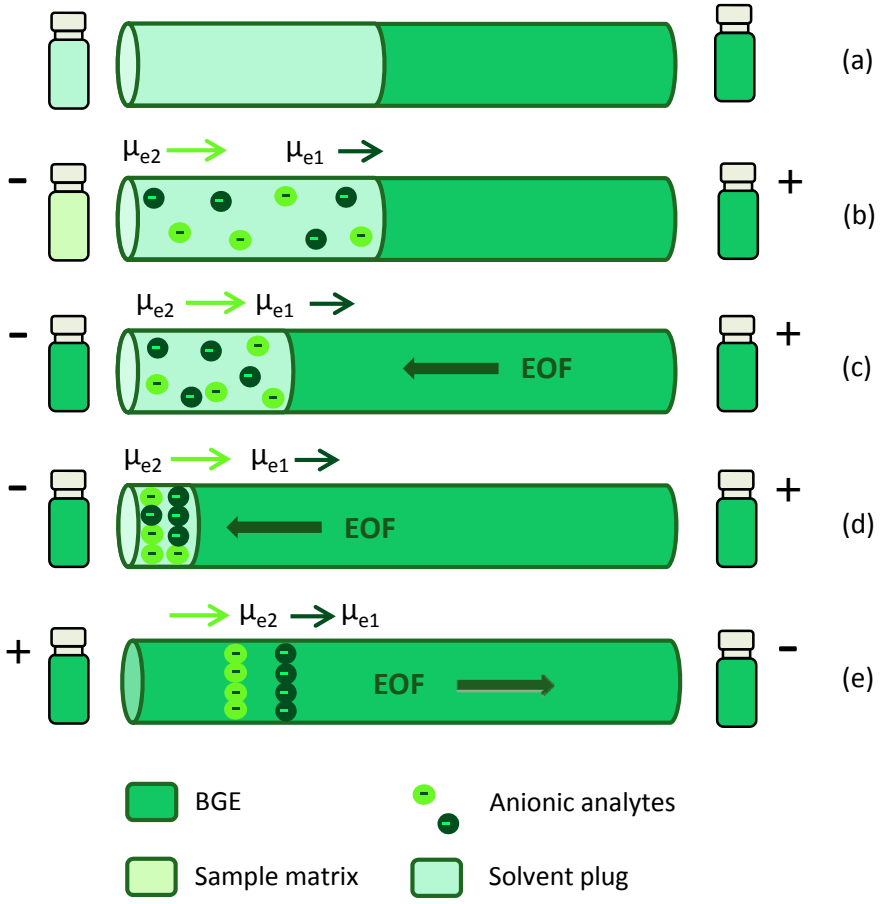

Figure 5. Schematic representation of *Field-Enhanced Sample Injection* (FESI) in CZE for the analysis of anions.

applied across the capillary. Because of the higher electric field strength in the solvent plug zone, the anionic analytes are easily injected electrokinetically through the solvent plug (b) and, simultaneously, the EOF pushes the solvent plug towards the inlet while the anionic analytes move quickly toward the outlet and they are accumulated or stacked at the interface between the solvent plug and the BGE (c). The current of the system increases in absolute terms as the capillary is filled with the BGE that comes from the outlet and the solvent is removed through the inlet. When the current reaches 95-99 % of the current value that exists when the capillary is totally filled with the BGE, the polarity is switched (d). With a positive voltage, the electrophoretic separation begins. The analytes migrate towards the detector due to the EOF mobility, since this magnitude is greater than the electrophoretic mobility of the anionic analytes (μ_{e1} and μ_{e2}) (e).

Li *et al.* [173] developed a novel method based on off-line hollow fibre based liquid-liquid-liquid microextraction (HF-LLLME) combined with FESI for the preconcentration and determination of five phenyl arsenic compounds in pig feed from a local pig farm, and storage pig litter, soil in agricultural field and lake water collected near the pig farm. The HF-LLLME-FESI-CE method achieved LODs in the range of 0.68 ng mL^{-1} to 6.9 ng mL^{-1} and the improvement in detection sensitivity ranged from 155- to 1,780-fold for standard samples. Hai *et al.* [174] developed a rapid method with FESI for in-capillary derivatization to determine selenomethionine (SeMet) and selenomethionine selenoxide (SeOMet). The derivatization reagent phthalic anhydride was introduced hydrodynamically into the capillary, while the sample solution was injected electrokinetically, thus allowing a selective preconcentration of the analytes by FESI. The LODs were found to be 0.2 mM for SeOMet and 0.5 mM for SeMet and an improvement in detection sensitivity of about 800-fold was achieved for standard samples.

In 2001, Zhu and Lee [175] developed a method in which the sample was electrokinetically injected and, in this case, the sample matrix was removed by using the EOF pump without polarity switching. They called this approach *Field-Amplified Sample Injection with Matrix Removal via an EOF Pump* (FAEP). This technique has also been named *Field-Enhanced Sample Injection with Reverse Migrating Micelles* (FESI-RMM) and *Field-Enhanced Sample Injection with Reverse Migrating Pseudostationary Phase* (FESI-RMP), when applied to MEKC or MEEKC, respectively.

In the bibliography, there are very recent applications of this type of sample stacking techniques, which can provide up to a 2,500-fold improvement in sensitivity [160,162,167,169-171,176-178]. For instance, these techniques have been used to determine illegal drugs in urine samples [169], arginine and methylated metabolites in human plasma [170,176], herbicides in environmental waters [160,167], sorbic and benzoic acids in soy sauce [162], and nerve agent degradation products in river water and aqueous extracts of soil [178], among others. A method based on non-gel sieving CE (NGS–CE) with FAEP was developed for the separation and preconcentration of multiplex polymerase chain reaction products of three pathogenic bacteria in which hydroxypropyl methylcellulose was used as the sieving medium and dynamic capillary coating [177]. The ion pair reagent of tetrabutylammonium phosphate (TBAP) was used in NGS–CE to improve the resolution of DNA fragments and also as a reverse EOF agent to apply the FAEP preconcentration technique. Xu *et al.* [167] developed a solvent-bar microextraction combined with FESI-NACE to extract, preconcentrate and determine herbicides in river water samples. The achieved limits of detection were between 0.08

ng mL^{-1} and 0.14 ng mL^{-1} for the studied herbicides in standard samples. Hou *et al.* [171] developed a method for the simultaneous stacking of cationic and anionic compounds in a single run by two-end field-amplified sample injection. Following the capillary-filling with a high conductivity buffer, a water plug was loaded into each end of the capillary and two high-field strength zones were generated at both heads of the column when high voltage was applied. Therefore, under suppressed EOF, cations and anions can be selectively FASI stacked at anode and cathode head, respectively. After separation, the stacked anions and cations were detected by a UV detector placed in the centre of the capillary. Under the optimized conditions, the LODs for the model cationic (matrine and oxymatrine) and anionic (5-sulphosalicylic acid) compounds were determined as 0.2, 0.2 and 0.06 ng mL^{-1}, respectively, and the sensitivities of these compounds were enhanced 1,003-, 1,330- and 1,380-fold, respectively.

1.2.3. Sweeping

Sweeping is a sample preconcentration technique that was introduced by Quirino and Terabe in 1998 [179]. It is based on the preconcentration of analytes by an additive present in the BGE (i.e. pseudostationary phase (PS) or complexing agent) that penetrates into the sample zone that does not contain that additive. Thus, when the additive present in the BGE penetrates the sample zone during the application of voltage, the picking or accumulating of analytes occurs due to chromatographic partitioning, complexation or any interaction between the analytes and the additive. The level of focusing is dependent on the strength of the interaction involved. Furthermore, the sweeping preconcentration is independent of the EOF. The sample conductivity can be lower, similar or higher than the BGE conductivity, and while there is some inconsistency within the literature as to the name of this approach when high or lower conductivity samples are used, they will all be referred to as sweeping in this Doctoral Thesis.

The most common protocol for sweeping neutral and ionic analytes is based on using anionic micelles of sodium dodecyl sulphate (SDS) and suppressing the EOF by using a BGE at low pH provided by a phosphate buffer (pH 2-3) [6,180]. Figure 6 shows a schematic representation of this technique. After the conditioning of the capillary with the micellar low pH BGE, a large volume of sample, in which analytes are prepared in a matrix with the same conductivity as the BGE but free of micelles, is injected hydrodynamically into the capillary (a). Subsequently, the BGE is placed in the inlet vial and a negative voltage is applied, and then the anionic micelles enter the cathodic end

of the capillary (b). The advancing micelles begin to associate with the analytes in the sample zone. Simultaneously, a zone without micelles develops at the interface between the sample zone and BGE zone (c). The analyte-micelle complexes advance through the sample zone until they reach the boundary between sample plug and BGE. At this point the analytes are totally swept (d). The analytes migrate towards the detector by the electrophoretic mobility of the analyte-micelle complex (μ_{eM1} and μ_{eM2}), since the EOF is suppressed (e).

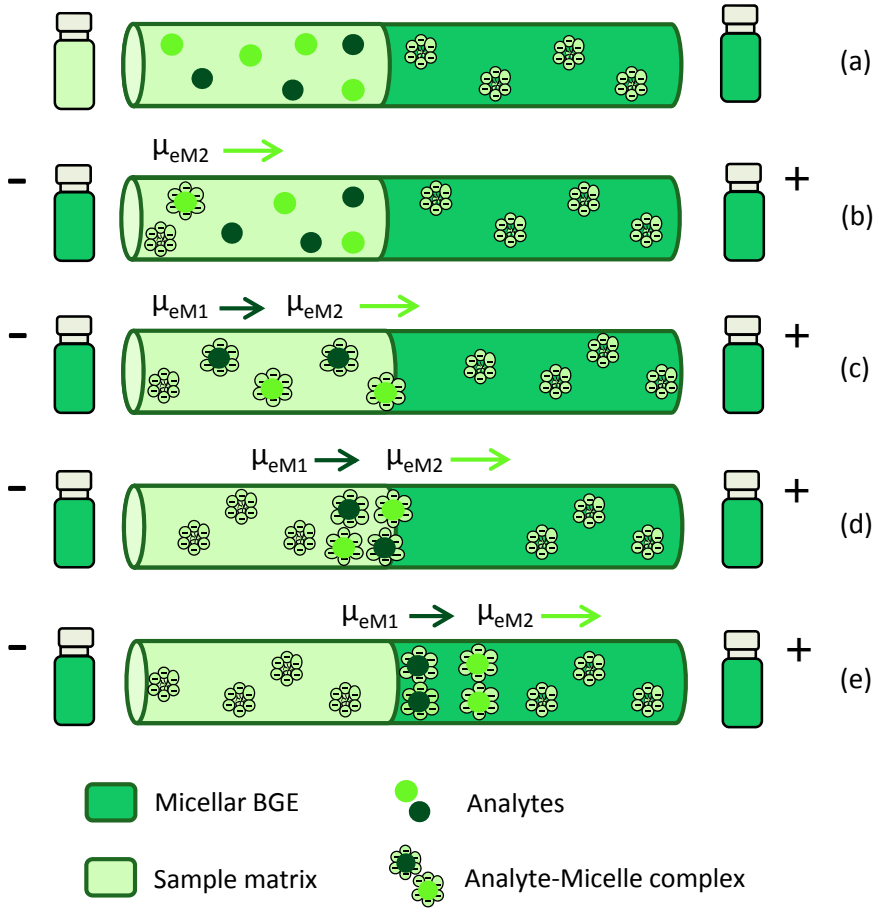

Figure 6. Schematic representation of Sweeping.

In general, the detection sensitivity using this sweeping protocol can be improved by between 10-fold and 1,500-fold [181-192] and, among the different applications reported in the literature, sweeping has been used to determine catecholamines and their metabolites [181], nitroaromatic explosive residue in soil samples [182], melamine in infant formulas [183], aromatic amines in water samples [184], oestrogens in water

samples [185], tetrahydrocannabinol and its major metabolites in urine samples [186], drugs of abuse and hypnotics in urine samples [187], biogenic amines in plasma samples and urine samples [188], hypolipidaemic drugs in wastewater samples [189], flavonoids in *Fructus aurantii immaturus* [190], triazole fungicides [191], and food grade antioxidants in food and vitamin samples [192]. For example, Tsai *et al*. [183] carried out a comparative study between FASS and sweeping. The two preconcentration techniques were compared in terms of their effectiveness in melamine determination in milk samples. Although the method including FASS provided better concentration efficiency (430-fold), the matrix effect was more prominent when it was applied to real samples. On the other hand, in the case of sweeping (70-fold), this matrix effect was almost negligible. The applicability of the established sweeping-MEKC method was demonstrated by analysing the milk samples.

In the case of sweeping in basic conditions (using borate buffer as the BGE at pH values in the range of 8.5 to 10), SDS is the most commonly used PS [6,180], and under these conditions, the migration of the analytes to the detector is mainly due to the EOF. The achieved enhancement in sensitivity is in the range 12-fold to 1,100-fold [193-201]. For instance, sweeping under basic conditions has been used to preconcentrate picoxystrobin and pyraclostrobin in urine samples [193], steroids in yeast mediated stereoselective reduction culture [194], carbamate pesticides in juice samples [195], aflatoxins in rice samples [196], three neutral steroids in urine samples [197], the antiviral drug oseltamivir and its hydrolyzed product in Tamiflu capsules [198], enantiomers compounds such us polycyclic musks enantiomers in personal care products [199], and aspartic acid enantiomer in cerebrospinal fluid, soy milk and beer [200]. For example, Xia *et al.* [201] combined an in-tube solid-phase microextraction and sweeping in basic conditions for the preconcentration of hydrophobic compounds (loratadine, indomethacin, ibuprofen and doxazosin). The developed method achieved enrichment factors of up to 1,355-fold and was applied for the determination of loratadine in rabbit blood.

As reported, SDS is the most commonly used additive in sweeping in both acidic and basic conditions. However, the use of cationic surfactants as sweeping carriers has also been reported [6,180]. The basic concept behind sweeping and separation using a cationic surfactant is the use of a basic BGE and a reversed-polarity voltage. When a cationic surfactant is added to the BGE, the capillary wall will be dynamically coated by a surfactant and, with a sufficient surfactant concentration, the EOF will be reversed and so reversed polarity voltage is required. The most commonly used cationic surfactants are dodecyltrimethylammonium bromide (DTAB) and CTAB. Some of the

compounds that have been determined under these conditionsinclude oligonucleotides, 6-carboxyfluorescein and fluorescein, and flunitrazepam and its major metabolites [202-204]. Recently, a sweeping method for the determination of benzodiazepines has been proposed with the use of ionic liquid (IL) type cationic surfactants [205]. ILs have received a great deal of attention for their use in separation science, and have been applied in recent years as additives for improving MEKC-based separations because of their high conductivity, hydrophobicity and solvating properties. The IL type cationic surfactants 1-cetyl-3-methylimidazolium bromide and N-cetyl-N-methylpyrrolidinium bromide resemble the commonly employed cationic surfactant CTAB, but provide different separation efficiencies. Using these IL type cationic surfactants, the sensitivity enhancement for the benzodiazepines were within the range of 31- to 165-fold [205]. Another reported strategy involves combining surfactants with different characteristics, as it was carried out by Cao *et al.* [206]. In particular, the authors investigated the possibility of using a mixture of anionic surfactants (SDS) and cationic surfactants (dodecyltrimethylammonium chloride (DTAC)) in sweeping combined with MEEKC for the determination of flavonoids. The detection sensitivity was improved from 18-fold to 508-fold, and the method was applied for the simultaneous quantification of five compounds in *Radix Astragali*.

As mentioned, the additive present in the BGE can be a PS (e.g. SDS) or a complexing agent. Sweeping via an additive that complexes with the sample in CZE has been employed to further enhance band narrowing and improve the focusing of a large volume of sample in the capillary. CZE is used mainly for the separation of charged analytes. However, with the aid of a complexing agent such as borate, neutral analytes containing vicinal diols can be separated. Borate anions interact with the cis-diol sites of the analytes to produce anionic or zwitterionic complexes, thus modifying the electrophoretic mobility. The complexing agent then serves as the carrier for sweeping and separation in CZE [180]. When a potential is applied, borate enters the sample zone and forms complexes with the analytes and the analyte-borate complex then accumulates in a concentrated zone. Recently, Cao *et al.* [207,208] reported a novel preconcentration technique based on dual sweeping for the direct analysis of neutral analytes. The basic principles behind this approach is that the neutral analytes converted to anions by the borate complexation are focused at the solvent viscosity difference junction (i.e. the difference in the solvent viscosities of the sample and BGE). They are then swept again by the Brij-35 micelles and then, finally, the separation in the non-ionic MEKC mode takes place. This approach has been applied to the analysis of neutral glucosides in the crude extracts obtained from plant samples, obtaining between 50-fold and 130-fold improvement in detection sensitivity. Metal ions can also

be separated and preconcentrated by sweeping via dynamic complexation with ethylenediamine tetraacetic acid (EDTA). EDTA forms strong 1:1 complexes with most metal ions, producing an anionic chelate, and thus altering the electrophoretic mobility of the analyte. EDTA acts as an on-line complexing agent and a carrier for sweeping. Using this strategy, the peak heights of several metal ions were improved up to 1,840-fold [209,210]. Copper ions were also employed as a complexing agent in order to complex amino acids for their determination in human saliva and green tea. Similarly to EDTA, copper ions act as an on-line complexing agent and carrier for sweeping [211].

Over the last decade, sweeping has been combined with other preconcentration techniques to further increase detection sensitivity. The first technique that was developed involved the combination of sweeping and stacking protocols. The resulting approach, named *Cation Selective Exhaustive Injection-Sweeping* (CSEI-sweeping), was first reported by Quirino and Terabe [212] and is based on a selective electrokinetic injection (SEI) of the sample followed by the sweeping process. This strategy was applied for cationic analytes, using SDS as the sweeping carrier, and an increase in the detection sensitivity of up to a million-fold was achieved [180,212]. This methodology has been mainly focused on the determination of drugs and their metabolites in biological samples. For instance, cocaine and its metabolites [213], heroin metabolites [214], N,N-dimethyltryptamine and related compounds [215], morphine and its main four metabolites [216], and methadone and its metabolites [217] have been determined in human urine. Furthermore, CSEI-sweeping has also been used to improve sensitivity in the analysis of the selective serotonin reuptake inhibitors of antidepressant drugs and isoniazid in human plasma [218,219], cypromazine and melanin in milk samples [220], tobacco-specific N-nitrosamines in urine samples [221], melanin and its derivates in milk samples [222], and malachite green in fishpond water samples [223], among others.

When anionic analytes are injected electrokinetically, the preconcentration approach is known as *Anion Selective Exhaustive Injection-Sweeping* (ASEI-sweeping) [224]. This is a more recent approach than CSEI-sweeping and the principle of the techniques is basically the same, although some modifications have been added. The application of ASEI-sweeping for anionic analytes is less widespread than the CSEI-sweeping, but an increase in detection sensitivity of over 100,000 fold has been reported [180,212,224]. In ASEI-sweeping, the focusing of anionic analytes can be carried out by cationic micelles, such as cetyltrimethylammonium chloride (CTAC) and DTAB, or anionic micelles, such as SDS. In the case of using cationic micelles, the polarities for injection and separation are reversed with respect to CSEI-sweeping: the anionic sample solution is injected electrokinetically at negative polarity and the separation is applied at positive

polarity. Aromatic carboxylic acids and fluorescein have been determined using this protocol [224,225].

SDS has been widely used as a surfactant in ASEI-sweeping procedures. It has also been used in MEKC as a MEEKC separation mode, and the preconcentration and the separation step is performed without switching polarity [226-230]. Figure 7 shows the schematic diagram of the preconcentration of anionic compounds by ASEI-sweeping using SDS. Firstly, the capillary is filled with an acidic micellar BGE solution in order to suppress the electroosmotic flow, followed by the hydrodynamic injection of a high-conductivity buffer (HCB) and, finally, by the hydrodynamic injection of a short plug of water (a). Then, using electrokinetic injection (at negative polarity), the anionic analytes enter the capillary through the water plug at high velocities (b) and are then focused or stacked at the HCB zone (c). The injection is then stopped and the BGE is placed in the inlet vial. A negative voltage is then applied, thus permitting the entry of micelles from the cathodic vial into the capillary (d). The micelles sweep the stacked analytes into a narrow band (e). Finally, the analytes migrate towards the detector by electrophoretic mobility of the analyte micelle complex (μ_{eM1} and μ_{eM1}), as the EOF is suppressed (f). The water plug during the SEI step helps to maintain a high electric field at the tip of the capillary, which allows the anionic analytes to enter the capillary at high velocity and eventually improves the stacking effect. Without the water plug, the analytes stack at the injection point, degrading the field enhancement. The presence of the HCB improves the total stacking effect of the analytes. It allows the injection of sample ions over a long period of time (as long as 1,000 s). The HCB increases the amount of sample injected and creates a narrower stacked zone after the SEI step. Both the water plug and the HCB improve the SEI step by creating a long sample zone with a higher concentration than in the original solution, but they do not contribute to the focusing effect of the analyte via sweeping [180]. ASEI-sweeping with SDS has been used to determine penicillins in groundwater [226], flavonoids in plant extracts [227], gallic acid and catechins in food [228], and acidic compounds [229]. Recently, Zhang *et al.* [230] developed an ASEI-sweeping method for the preconcentration and separation of neutral compounds under controlled EOF. The EOF was used to balance the electrophoretic migration of SDS micelles to generate an immobile sweeping boundary, allowing the injection of a large volume of sample. Sample solution was injected into the capillary for 70 min and the detection sensitivity was improved more than 20,000-fold. The proposed method has been applied to analyse trace phlorizin and quercitrin in urine samples.

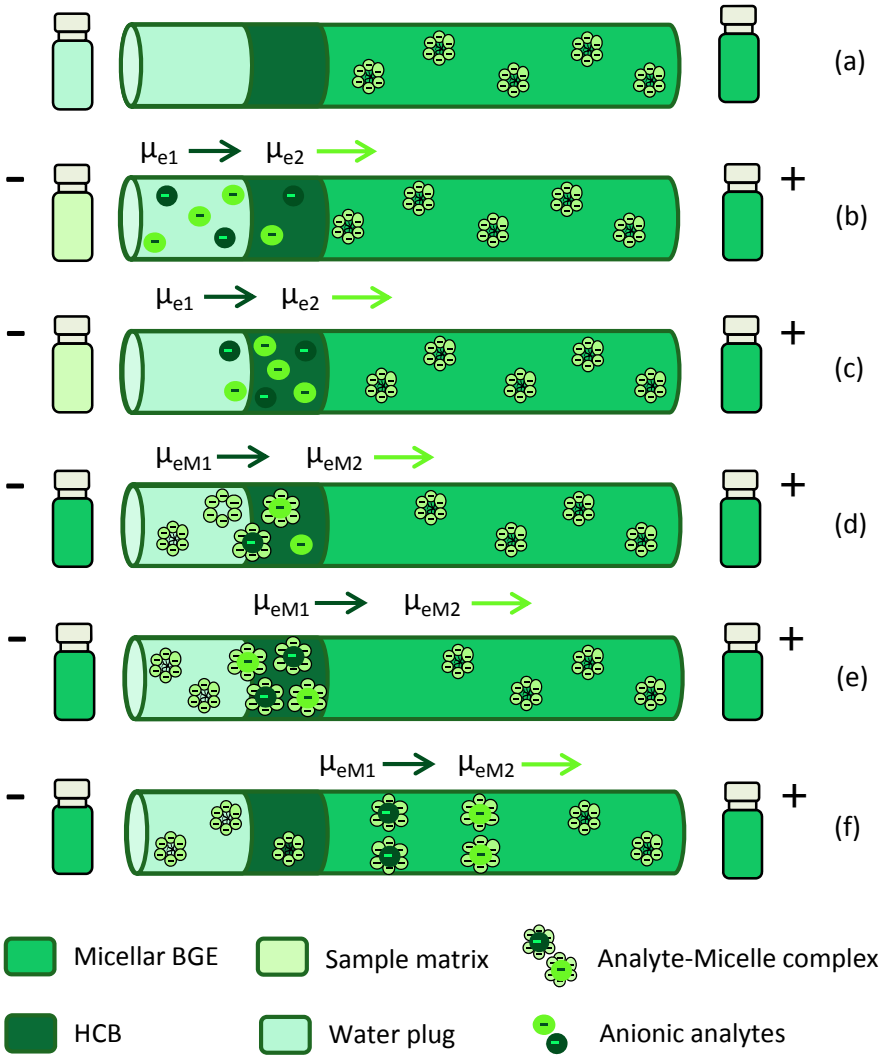

Figure 7. Schematic representation of Anion-Selective Exhaustive Injection-Sweeping (ASEI-sweeping).

Sweeping has also been combined with LVSS, resulting in an easy and simple preconcentration technique. This technique was carried out with the hydrodynamic injection of a large volume of sample, which is prepared in a low-conductivity solution. Then, with the application of a positive voltage, the sample plug is pumped towards the outlet end of the capillary by EOF, while the anionic analytes contained in this low-conductivity plug are stacked at the boundary between the sample matrix and the BGE. The continuous application of a positive voltage allows micelles to migrate into the sample zone where they sweep the analytes that are already stacked. Zhang *et al.* [231]

introduced LVSS-sweeping to determine polyphenols in tea. Under optimal preconcentration conditions, the enrichment factors of peak height and peak area of the polyphenols were in the range of 18- to 26-fold and 23- to 44-fold, respectively. Zhu *et al*. [232] developed a LVSS-sweeping methodology to determine adenine, caffeine, theophylline, reduced L-glutathione and oxidized L-glutathione in two different teas. The enrichment factors of peak height and peak area of the analytes were in the range of 9 to 33 and 21 to 35 respectively, and the LODs ranged from 26.5 ng mL^{-1} to 55.8 ng mL^{-1} for standard samples. The same authors [233] developed an on-line combination of single-drop liquid-liquid-liquid microextraction (SD-LLLME) with LVSS-sweeping for sample clean-up and preconcentration, and the enrichment factors with the SD-LLLME-LVSS-sweeping were improved up to 550-fold and the LODs achieved were 2 ng mL^{-1}. To evaluate the practical applicability of the developed method, it was applied to the analysis of adenine in green tea.

Recently, a dual dynamic pH junction-sweeping focusing method has been reported [234-237]. Dynamic pH junction-sweeping is performed when the sample is devoid of micelles (sweeping conditions) and has a different pH from the BGE (dynamic pH junction conditions). This hyphenated focusing approach aims to improve the focusing performance of conventional sweeping or the dynamic pH junction for neutral and weakly ionic species [236]. This technique has achieved up to 5,500-fold improvement in detection sensitivity, and has been used to preconcentrate and determine phenolic acids in Majorana hortensis leaves [235], benzoic acid in sunflower oil [236], and methotrexate and its metabolites in CSF samples [237]. Chen *et al*. [234] have developed a dynamic pH junction-sweeping method using a LIF detector, in which the focusing effect was induced by several distinct processes, including buffer pH, micelle partitioning and borate complexation. The proposed method was used to determine dipeptides in human serum sample. The detection limits of the four dipeptides were in the range of 1.0 pmol L^{-1} to 5.0 pmol L^{-1} for standard samples.

1.2.3. In-line solid-phase extraction-capillary electrophoresis

Chromatographic preconcentration techniques represent another interesting strategy for increasing the sensitivity of CE. These techniques were developed later than the previously discussed techniques. In chromatographic-based preconcentration techniques, analytes from a large volume of sample are concentrated on a sorbent and then eluted in a small amount of solvent, leading to lower detection limits [8-12]. As previously mentioned, among the chromatographic preconcentration techniques

coupled to CE, SPE is the preferred choice and, in particular, the in-line SPE configuration is the most widely used [8-12]. In this approach, an analyte concentrator (AC) is incorporated into the CE capillary where the separation takes place, giving a high degree of integration.

The main advantages of the in-line coupling between SPE and CE are that the design does not involve the modification of the CE system, being easily automated with fewer sample-handling steps, requires low volume of organic solvent consumption throughout the process, needs a relatively small quantity of sorbent material for the construction of the SPE device, and is capable of analysing the complete eluate by CE. In spite of these great benefits, the in-line SPE-CE is not a widely used technique as ACs are not commercially available and need to be constructed by the user. Moreover, when complex samples are analysed, problems with clogging can occur in addition to irreproducible results caused by the adsorption of sample matrix components by the capillary walls [8-12].

In-line SPE-CE was first developed by Guzman *et al.* [238] in 1991. In this approach, an AC containing an antibody covalently bound to a solid support material is inserted into the inlet section of the electrophoretic capillary, allowing the retention and concentration of the analytes before the electrophoretic separation takes place. The developed method was used to preconcentrate and separate a group of urinary compounds.

The different steps followed to preconcentrate and separate the analytes by an in-line SPE-CE system are shown in Figure 8, and are the usual steps involved in any SPE procedure. In this case, the diagram represents the preconcentration and separation of anionic analytes. Firstly, after the conditioning of the capillary with the BGE, the SPE sorbent is conditioned with the appropriate organic solvent and Milli-Q water at an adequate pH. Then, a large volume of sample is injected hydrodynamically and the analytes are retained in the SPE sorbent. This step is known as sample loading step (a). The washing step is carried out with with BGE (b), which is followed by the elution of the retained analytes by hydrodynamically injecting a plug of elution solvent, known as the elution plug (c). Then this plug is pushed through the concentrator until the beginning of the separation capillary by a hydrodynamic injection of BGE, known as the pushing step (d). Finally, the separation voltage is applied across the SPE material to carry out the separation of the analytes by CE (e).

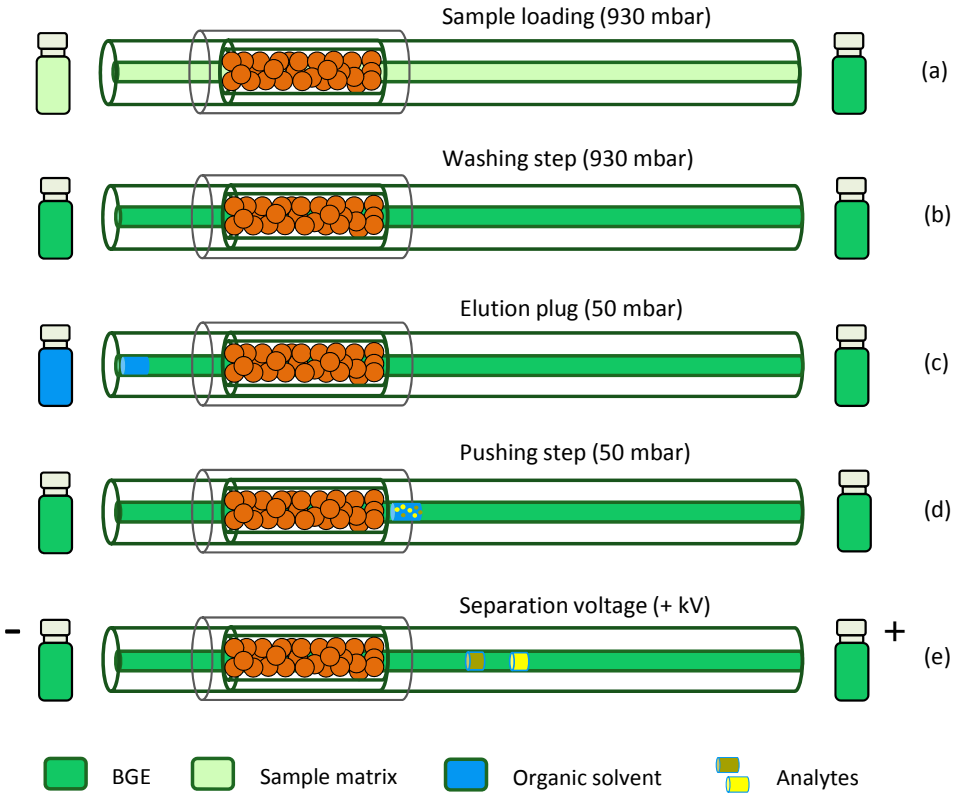

Figure 8. Schematic representation of the different steps involved in in-line SPE-CE for the analysis of anions.

Since its introduction, several devices have been described for in-line SPE-CE. In general, in-line SPE-CE systems can be divided into three typical designs. The AC can be (i) an open-tubular (OT) capillary that is coated with the SPE sorbent, (ii) a packed bed or monolithic material in a capillary, or (iii) a thin impregnated membrane or SPE material positioned between two capillaries. More details on the general design of in-line SPE systems can be found in recent reviews [8,9]. This Doctoral Thesis has focused on packed bed or monolithic material in a capillary, as most of the published papers in recent years have concentrated on these kinds of devices. Figure 9 shows a schematic representation of the most commonly used packed bed designs for the in-line SPE-CE system. Table 2 shows an overview of the in-line SPE-CE studies published between 2008 and March 2012 based on this kind of design.

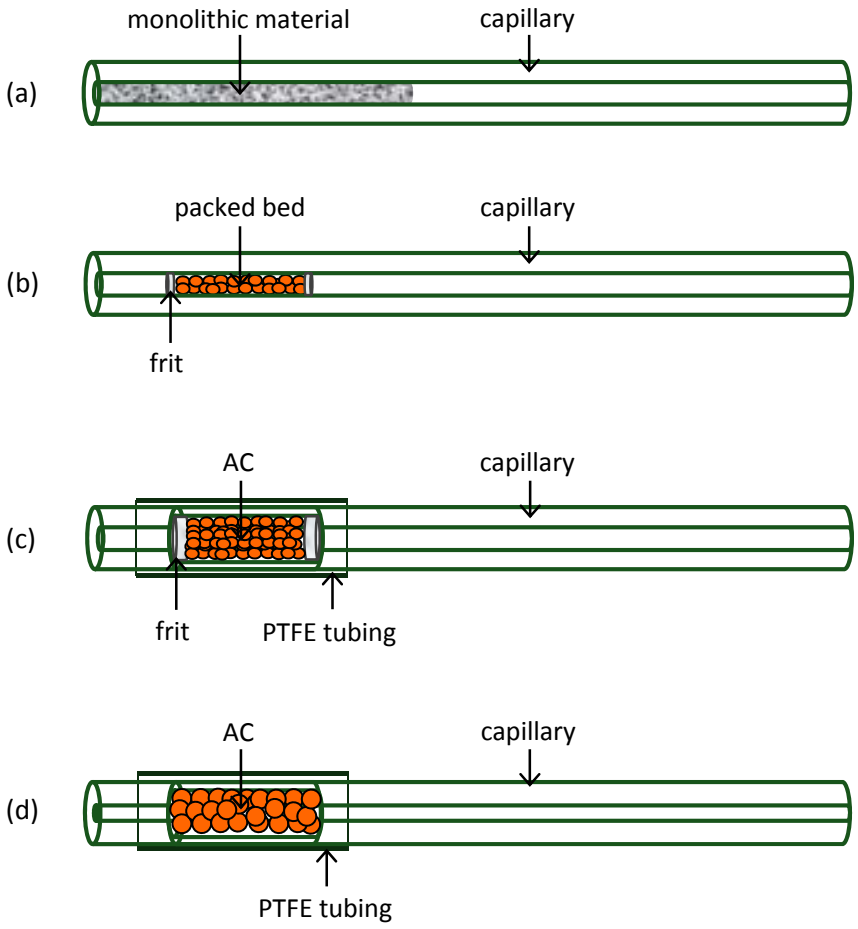

Figure 9. Schematic representation of the packed bed designs: (a) Packed bed monolithic material inside the separation capillary; (b) Packed bed SPE sorbent inside the separation capillary retained by frits; (c) Packed bed SPE sorbent positioned between two capillaries inside an AC and retained by two frits; (d) Packed bed SPE sorbent positioned between two capillaries inside an AC without frits.

In the packed bed designs, there are different possible AC constructions. One option is that a piece of monolithic material (also known as continuous beds) can be synthesized inside the electrophoretic separation capillary (Figure 9a). Another possibility is to keep the packing material inside the electrophoretic separation capillary with retaining frits (Figure 9b). The final strategy is to use a SPE material to construct an AC and couple it in-line between two capillaries with frits (Figure 9c), and without frits (Figure 9d). Generally, the packed beds are constructed from sorbent particles extracted from a commercial SPE cartridge.

Table 2. Overview of in-line SPE-CE applications reported between 2008 and March 2012.

Analyte	Sample	SPE sorbent	LOD (ng mL^{-1})	Ref.
Methionine enkephalin	Cerebrospinal fluid	Monolithic sol-gel column	1.0	[239]
Weak bases	Water	Poly(MA-co-EGDMA) Monolith	8.0-30	[240]
Neurotransmitters	Human urine	Silica nanoparticle-templated monolith	0.5-0.7	[241]
Carbamate pesticides	Drinking water	Divinylbenzene-based monolithic polymer	0.01	[242]
TER[a] HMMA[b]	Run buffer	C18	2.5-15	[243]
Parabens & NSAIDs	Aqueous sample	Functionalized magnetic silica-coated iron oxide particles	n.s.	[244]
Peptides	Human plasma	C18	10-1.0 *	[245]
Peptides	Human plasma	C18	0.1-1.0	[246]
Rare earth metals	Tap water	C-18-derivatized silica particles	20-80 pg L^{-1}	[247]
Ochratoxin	River water	C18	1,000	[248]
Naproxen	Tap water	C18	0.2	[83]
Drugs of abuse	River and tap water	Oasis HLB	0.05-0.20 0.07-0.26 *	[249]
Drugs of abuse	Human urine	Oasis HLB	0.008-0.115 0.013-0.210*	[250]
Sulphonamides	Tap, bottled and river water	Oasis HLB	0.3-0.6	[251]
Quinolones	Meat	Oasis MCX	0.017-0.059	[252]
Recombinant human EPO	Aqueous solution	Immuno-affinity column based on polyclonal anti-EPO antibody	~ µg mL^{-1}	[253]
Triazine herbicides	Human urine	Molecularly imprinted polymer	200-600	[254]
Peptides	Human plasma	C18	0.01-0.1	[255]

[a] Terbutaline

[b] 4-Hydroxy-3-methoxy-methamphetamine

n.s.: not specified in paper

* LODs in real samples

Monolithic columns are a new generation of chromatographic stationary phases that are highly promising for in-line SPE-CE. Monoliths can be synthesized directly inside the capillary and anchored to the wall through chemical bonding, removing the need for frits. The monolith itself consists of a rigid macroporous structure which can be prepared by polymerization of a precursor mixture in situ [256,257]. The first application involving the use of a monolithic polymer as a preconcentrator was demonstrated by Baryla and Toltl in 2003 [258], who prepared a 1 cm long methacrylate monolith at the inlet end of a capillary column by photoinitiated polymerization for the preconcentration of S-propranolol.

To date, two types of monoliths have been introduced, namely silica-based monoliths and polymer-based monoliths [256,257]. Silica monoliths are made through the condensation of alkylsilanes via sol–gel chemistry, while polymer-based monoliths (including acrylate, methacrylate, acrylamide and styrene) are prepared by polymerizing monomers and crosslinkers in the presence of porogens. Silica monoliths have very high surface areas due to the presence of mesopores within the skeleton structure, but cannot be used under basic conditions due to the dissolution of the monolith [239]. Porous polymer monoliths are not subject to this limitation and are therefore a highly attractive option, particularly as they can also be prepared directly within the capillary by photoinitiation, allowing the exact position of the monolith to be controlled. However, they have a low surface area compared to silica monoliths. This lower capacity may be problematic for the SPE of real samples containing complex sample matrices where high concentrations of competing ions are present [240-242].

Despite the advantages of monoliths as preconcentration sorbents, they are still not widely used for in-line SPE-CE of different analytes in real samples. To date, there are only a few papers in the literature which show the applicability of this kind of material in real samples [239-242]. However, it should be pointed out that monolithic materials are very promising for the exploration of in-line SPE-CE applications. Ramautar *et al*. [239] evaluated a monolithic sol–gel concentrator of 5 mm for in-line SPE-CE-ESI-MS analysis of methionine enkephalin in CSF. A 40-fold preconcentration was obtained for a methionine enkephalin test solution using a loading volume of 3,200 nL, and the LOD was 1 ng mL^{-1} (ca. 5 nM) for methionine enkephalin in CSF. Thabano *et al.* [240,241] developed two studies based on the use of porous polymer monoliths. In the first study [240], the authors developed an in-line SPE-CE using a methacrylate-based weak cation-exchange poly(MA-co-EGDMA) monolithic stationary phase of 8 mm in conjunction with a novel moving pH boundary in order to elute the retained analytes from the extraction phase. Neurotransmitters were used as test analytes, achieving a 35-fold improvement

in detection sensitivity. In the second study [241], the ion-exchange capacity of the methacrylate polymer monoliths was increased by templating the monoliths with silica nanoparticles. Neurotransmitters were also used as test analytes, achieving up to 1,900-fold improvement in detection sensitivity. The advantage of the higher capacity monolith was demonstrated for the analysis of a human urine sample without any sample pretreatment. Rodríguez-Gonzalo *et al.* [242] developed an in-line SPE-CE method with a divinylbenzene-based monolithic polymer of 5 mm in length for the preconcentration and separation of two carbamate pesticides, pirimicarb and carbendazim, in drinking water samples. The retained compounds were desorbed from the monolithic column with the same BGE used for their separation by MEKC. The detection sensitivity was improved up to 19,000-fold, and the developed in-line SPE-CE method allowed the determination of these pesticides in drinking water at a concentration level of 0.1 ng mL^{-1}.

Designs which involve packing the SPE material inside the electrophoretic separation capillary by retaining frits present difficulties when building the system, as well as the problems caused by the presence of frits. The use of frits can lead to increases in backpressure, long analysis times and irreproducible EOFs. Moreover, continuous use can result in compression of the column bed, which leads to irreproducible flow rates [8,259]. Despite these facts, the study carried out by Chaisuwan *et al*. [243] should be noted, in which C18 sorbent was packed inside the separation capillary using frits. With this in-line SPE-CE system, 4-Hydroxy-3-methoxy-methamphetamine (HMMA) and terbutaline were preconcentrated, achieving up to 1,000-fold improvement in detection sensitivity. Another study that used a packed bed design inside the separation capillary was carried out by Tennico *et al.* [244], which used magnetic particles as a new SPE material. In this study, silica-coated iron oxide particles were synthesized and used as the solid support. The particles were functionalized with octadecylsilane and used as reversed-phase sorbents for in-line SPE-CE. Magnets were used to immobilize the magnetic SPE particles locally inside the capillary. A mixture of hydrophobic test compounds (parabens and NSAIDs) was analysed to demonstrate the efficacy of the magnetic particles in the in-line SPE-CE process.

Of all of the packed bed configurations, the most popular device is the one which uses a SPE material to construct an AC and couples it in-line between two capillaries. The AC is constructed by using a small piece of capillary, which generally has a length of between 2 mm and 1 cm, and internal diameters (I.D.) (150-250 μm) higher than the I.D. of the separation capillary (50-75 μm). The SPE material can be retained in the AC by means of two frits in order to prevent the sorbent particles from entering and blocking the

separation CE capillary. Alternatively, it can be retained without frits through the use of sorbent particles with higher sizes than the I.D. of the separation capillary. The most commonly used protocol for constructing an AC is by using frits. However, as previously mentioned, frits can give rise to certain problems, especially when high resolution is needed [8,259]. The strategy to construct the frit-free concentrator was introduced by Saavedra *et al*. [260], which used Oasis MCX for the construction of a frit-free AC to determine 3-nitro-L-tyrosine in biological samples.

The protocol for building an AC consists of the following steps. Firstly, the appropriate length of bare fused silica capillary is conditioned and then cut into two pieces, one about 8 cm (this length depends on the CE instrument used) and the other depending on the length needed for the CE separation. The AC is prepared by cutting the appropriate length of bare fused-silica capillary, between 7 mm and 10 mm when frits are used and between 2 mm and 5 mm when frits are not used. A longer length is needed when frits are used as they occupy a volume of the AC. A clean cut in both sides of this capillary is essential in order to obtain optimal performance of the concentrator. The entire process of fabricating the AC is monitored under a microscope. The SPE sorbent is then loaded into the AC using a vacuum pump and a syringe. In the case of using frits, one frit has to be inserted into the AC before loading the sorbent and the second frit after filling the AC. Finally, the AC is inserted between the two pieces of capillary with the help of Polytetrafluoroethylene (PTFE) tubing.

The most commonly used sorbents for the construction of an AC are reversed-phase sorbents (C18 and Oasis HLB), and mixed-mode cation-exchange and reversed-phase sorbents (Oasis MCX). These have been applied to the preconcentration of different compounds in several matrices. The C18 sorbent has been used to construct an AC in an in-line SPE-CE system to determine peptides in human plasma [245-246], rare earth metals in tap water [247], ochratoxin in river water [248], and naproxen in tap water [83]. The Oasis HLB sorbent has been used in an in-line SPE-CE system for determining drugs of abuse in tap and river water and urine [249,250], and sulphonamides in tap, bottle and river water [251]. The Oasis MCX sorbent has been used in an in-line SPE-CE system for determining quinolones in chicken muscle samples [252]. Botello *et al.* [249,250] developed an in-line SPE-CE system using Oasis HLB to construct the AC for the preconcentration and determination of a group drugs of abuse. In the first study, the LODs reached for standard samples ranged between 0.05 ng mL^{-1} and 0.2 ng mL^{-1}, with sensitivity enhancement factors ranging from 2,300- to 5,300-fold. The applicability of the developed method was demonstrated in tap and river water samples which were directly analysed without any off-line pretreatment [249]. In the second study, the same

in-line SPE-CE system coupled with a MS detector was developed to preconcentrate and determine drugs of abuse in urine samples. The urine samples were only diluted 1:1 (v/v) before analysis and LODs achieved were between 0.013 ng mL^{-1} and 0.210 ng mL^{-1}. These are the lowest detection limits for those drugs in urine samples [250]. Lara *et al*. [251] developed an in-line SPE-CE-UV, using also Oasis HLB to construct the AC in order to determine a group of sulphonamides in tap, bottle and river water. No sample pretreatment was required and the LODs reached for the different water samples ranged between 0.3 ng mL^{-1} and 0.6 ng mL^{-1}. The same group [252] developed an in-line SPE-CE-MS/MS using sorbent particles of Oasis MCX to construct the AC in order to determine eight veterinary quinolones in chicken muscle. A pressurized liquid extraction (PLE) method was optimized to extract these antibiotics from the chicken muscle samples. The LODs obtained for the PLE-SPE-CE-MS/MS method ranged from 0.5 mg kg^{-1} to 20 mg kg^{-1}.

Furthermore, more selective sorbents have also been evaluated for the construction of in-line SPE-CE systems without frits. Giménez *et al*. [253] developed an in-line immunoaffinity (IA) CE-MS method in order to determine the recombinant human erythropoietin (rhEPO) by detection of a specific peptide marker. The IA stationary phase was prepared from a custom-made polyclonal anti-EPO (81–95) antibody immobilized on a solid support of CNBr-Sepharose and was packed in a AC of 0.7 cm near the inlet of the CE capillary. Although the limits of detection for the peptide marker were similar to those obtained with CE–MS (a few mg L^{-1}), the study shows the potential of this novel approach for selective and unambiguous detection in rhEPO and its analogues at the levels expected in biological fluids. Lara *et al*. [254] evaluated the use of a molecularly imprinted polymer (MIPs) as a sorbent for the in-line SPE-CE determination of triazine herbicides. The developed in-line MIP-SPE-CE-UV method achieved LODs ranging from 0.2 µg mL^{-1} to 0.6 µg mL^{-1} for standard samples. Furthermore, MIPs were compared with the Oasis HLB sorbent for the in-line SPE-CE analysis of the triazine herbicides in the urine samples. When Oasis HLB was used to analyse urine samples lower recoveries were obtained than those obtained with the MIP. This is probably due to the retention and competition of the many endogenous compounds from the matrix.

An interesting approach reported by Medina-Casanellas *et al.* [255] results from the combination of transient isotachophoresis with in-line SPE-CE with a MS detector (SPE-tITP-CE-TOF-MS) in order to improve the sensitivity of peptide analysis. Detection sensitivity was improved up to 1,900-fold. In that paper, the authors developed a methodology that combines the requirements of in-line SPE, tITP and MS for the

analysis of plasma samples. The LODs obtained in standard solutions and plasma samples for some of the studied peptides were 0.01 ng mL^{-1} and 0.1 ng mL^{-1}, respectively.

1.3. References

UNIVERSITAT ROVIRA I VIRGILI
PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
Irene Maijó Ferré
Dipòsit Legal: T. 1056-2012

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2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
Irene Maijó Ferré
Dipòsit Legal: T. 1056-2012

The main objective of this Doctoral Thesis is the development of different strategies to decrease the detection limits of capillary electrophoresis for the determination of pharmaceutical and personal care products. These strategies are based on electrophoretic and chromatographic preconcentration techniques, and the use of mass spectrometry as a detection system. The electrophoretic preconcentration techniques studied included sample stacking techniques and sweeping while the chromatographic preconcentration technique evaluated was in-line coupling between solid phase extraction and capillary electrophoresis. With respect to PPCPs, this Doctoral Thesis focuses specifically on non-steroidal anti-inflammatory drugs (NSAIDs), parabens and UV-filters.

Another objective of this Doctoral Thesis is to study the suitability of the developed methodologies for the determination of PPCPs in environmental water samples.

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3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As mentioned in the introduction to this Doctoral Thesis, PPCPs are a group of organic contaminants introduced in the environment, mainly in aquatic systems, as they are ineffectively removed from wastewater treatment plants. Therefore, the determination of water quality is of increasing concern because of the known adverse effects of some organic contaminants on human health and on the environment. To this end, the development of highly sensitive and selective methods for the determination of different PPCPs in environmental waters is of major importance within the scientific community.

Over recent years, the development of different preconcentration techniques applied to CE, in addition to the great versatility that the technique offers, has meant that CE has become a more promising candidate as an alternative to chromatographic techniques in the environmental field for the determination of organic contaminants. The research contained in this Doctoral Thesis was carried out in response to the growing interest in developing electrophoretic and chromatographic preconcentration techniques to increase the CE sensitivity for the determination of PPCPs, which are often found at very low concentration levels in environmental samples.

This chapter includes the experimental part, results and discussion from the different studies that have been carried out for this Doctoral Thesis. These results have already been published, or are in the process of being published, in several international scientific journals, and are presented in each section here in article format. These studies have been classified into three sections and, for each section, a brief introduction is included in order to establish the context of the research and also a discussion of the most notable results. The list of papers published as a result of this Doctoral Thesis is included in Appendix II.

In the first section, two methods for the determination of a group of NSAIDs in river water samples were developed. In both, electrophoretic preconcentration techniques were used to increase the sensitivity and MEKC was used as the electrophoretic separation mode. The electrophoretic preconcentration approaches developed were sweeping and ASEI-sweeping. Both methods were validated by analysing river water samples spiked with NSAIDs without the need for a previous sample pretreatment.

The second section focused on the use of a chromatographic technique to enhance the sensitivity of CE. To be specific, in-line coupling between SPE and CE was evaluated in two different studies. In the first, an in-line SPE-CE coupling was developed for the separation and preconcentration of a group of acidic pharmaceutical compounds. The SPE sorbent used to construct the analyte concentrator was a reversed-phase sorbent

(Oasis HLB). The method developed was applied to the analysis of river water samples spiked with the analytes. The aim of the second study was to evaluate the suitability of different sorbents for the preconcentration of UV-filters compounds through an in-line SPE-CE system with an MS detector. The three different sorbents evaluated and compared were: a reversed-phase sorbent (Oasis HLB), a mixed-mode anion-exchange and reversed-phase sorbent (Oasis MAX), and a mixed-mode cation-exchange and reversed-phase sorbent (Oasis MCX). The applicability of the developed in-line SPE-CE-ESI-MS methodology was demonstrated by the analysis of a group of UV-filters in river water samples. This study was carried out in collaboration with Professor Christian Neusüß of the Chemistry Department of the Aalen University (Germany), during my stay at that university.

In the last section, a comparative study between electrophoretic and chromatographic preconcentration techniques to enhance the sensitivity of CE was carried out. Two of the electrophoretic techniques selected were based on the sample stacking strategy applied to CZE: large volume sample stacking, which involves hydrodynamic injection of the sample, and field-amplified sample injection, which involves electrokinetic injection of the sample. The third electrophoretic technique was based on sweeping in MEKC. As a chromatographic technique, the in-line SPE-CE system with UV-Vis detector was used and the construction of the analyte concentrator was performed with a reversed-phase sorbent (Oasis HLB). The methods were investigated for the simultaneous preconcentration and separation of a group of parabens. Furthermore, the developed methodologies were validated with standard samples to show the potential of these preconcentration techniques for future applications to real samples.

All of the studies reported in this Doctoral Thesis were financially supported by the Ministry of Science and Technology (Projects CTM2008-06847-CO2-01/TECNO and CTM2008-0825) and by the Ministry of Science and Innovation (Project CTQ2011-24179).

3.1. Application of different electrophoretic preconcentration techniques in micellar electrokinetic capillary chromatography to determine non-steroidal anti-inflammatory drugs

UNIVERSITAT ROVIRA I VIRGILI
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As mentioned in the introduction, the widespread use of pharmaceutical compounds either for human or veterinary use generates continuous contamination of the aquatic system, as the pharmaceutical compounds and their metabolites are not efficiently removed by the conventional treatments in the wastewater treatment plants [1,2]. Over recent decades, there has been growing concern over the toxicity and the health effects that these organic contaminants may cause in the environment [3,4].

NSAIDs are a group of pharmaceutical compounds which have been detected in different aquatic systems, such as wastewater, surface waters and even drinking waters, at concentration levels between ng mL^{-1} and pg mL^{-1}. The development of suitable analytical methods for determining NSAIDs and their degradation products remains an area that is widely studied by many research groups today [1,2,5].

The research group in which I have carried out my Doctoral Thesis has previously studied the determination of NSAIDs in environmental waters by either chromatographic techniques [6,7] or by capillary electrophoresis (CE) [8-11]. Generally, the analysis of environmental water samples by CE has been performed along with a preconcentration technique coupled to CE and, whenever necessary, an off-line preconcentration or clean-up step has been performed in order to reach the low concentration levels at which these compounds are normally found in environmental waters.

The objectives of this section were focused on the use of preconcentration techniques in CE that had not previously been evaluated for determining NSAIDs in water samples, such as sweeping and sweeping combined with ASEI (ASEI-sweeping). The electrophoretic preconcentration technique of sweeping has demonstrated great preconcentration potential for certain compounds, achieving an increase of sensitivity of up to 1,500-fold [12]. In the first study, sweeping was evaluated as a preconcentration technique, and the main purpose was to find out the optimal conditions in terms of achieving the highest sensitivity. In that study, the main parameters affecting the preconcentration were the composition of the sample matrix and the sample injection time. Elsewhere in the literature, there are different studies which combined sweeping with other stacking methods to sweep zones that have already been stacked with the main result being a higher enhancement in sensitivity. With this in mind, in the second study, the combination of sweeping with a sample stacking technique was examined. In that case, the sample was electrokinetically injected. Firstly, preconcentration occurred through the sample stacking process and, subsequently, the analytes were focused by sweeping. The overall technique that was

applied is named anion-selective exhaustive injection-sweeping (ASEI-sweeping). As in the previous study, the main parameters involved in the preconcentration were optimized. Both the study into sweeping and the other into ASEI-sweeping were developed using SDS micelles as a PS and a BGE under pH-suppressed EOF. Working in a system with suppressed EOF enabled the separation and preconcentration of the analytes to be carried out without the need to switch the polarity between the preconcentration and separation steps.

The NSAIDs determined in both studies were ibuprofen, fenoprofen, diclofenac sodium, ketoprofen and naproxen. The structures of these NSAIDs are shown in the introduction chapter of this Doctoral Thesis. These compounds were chosen because they are all widely used and have been reported in the literature as being present in various types of water samples. Therefore, the developed methods were applied to the determination of this group of NSAIDs in river water samples from the Ebro River.

A paper with the results obtained in the first study has been accepted for publication in the *Journal of Liquid Chromatography & Related Technologies*. The second study has been published in *Chromatographia* 73 (2011) 83-91.

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3.1.1. Determination of anti-inflammatory drugs in river water by sweeping-micellar electrokinetic capillary chromatography

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DETERMINATION OF ANTI-INFLAMMATORY DRUGS IN RIVER WATER BY SWEEPING-MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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Abstract

This paper describes a simple and sensitive sweeping-micellar electrokinetic chromatography (sweeping-MEKC) method for the preconcentration and separation of five non-steroidal anti-inflammatory drugs (NSAIDs): ibuprofen, fenoprofen, naproxen, diclofenac sodium, and ketoprofen. We have optimized the sweeping conditions, including the composition of the sample matrix and the sample injection time. With this sweeping method, about 163- and 401- fold improvements in peak height and peak area, respectively, were obtained. The calibration plots were linear ($r^2 \geq 0.998$) over a range of 5-500 ng/mL for diclofenac, fenoprofen and naproxen and 10-500 ng/mL for ibuprofen and ketoprofen, and the detection limits were in the range of 1.4-2.5 ng/mL. Finally, to show the capability of the sweeping-MEKC method for the analysis of environmental aquatic samples, river water samples fortified with the NSAIDs compounds were analyzed without any previous sample pretreatment, obtaining limits of detection ranging between 8.4 and 14.6 ng/mL.

Keywords: micellar electrokinetic capillary chromatography (MEKC), non-steroidal anti-inflammatory drugs (NSAIDs), river water, sweeping.

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the group of analgesics and anti-inflammatory drugs most commonly used in human and veterinary medicine and often found in the aquatic environment. Owing to their hydrophilicity and stability, NSAIDs tend to remain in the aqueous phase and are not totally eliminated by the sewage treatment plants (STPs), and as a consequence they are frequently detected in waters. The analysis of these compounds in the aquatic environment involve a laborious task, associated with the complexity of the matrices and their

low concentration levels in water. The concentrations of individual compounds in surface waters are typically in the range of several tens to hundreds of ng/L, although concentrations at the μg/L level are also reported for some compounds and specific sites [1-5]. In this sense, very sensitive and selective analytical methodologies have been developed for their determination. The main techniques that have been used are high-performance liquid chromatography-tandem mass spectroscopy (HPLC-MS/MS) [4,6,7] and gas chromatography mass spectroscopy (GC-MS) [7-9]. CE combined with different preconcentration techniques has also been applied to determine these compounds in water samples [5,9-17]. In these previous manuscripts the main preconcentration techniques used have been based on stacking [5,13,14,16], electrokinetic super-charging (EKS) [10-12], anion-selective exhaustive injection sweeping (ASEI-sweeping) [9] and in-line SPE-CE [15,17].

Since micellar electrokinetic capillary chromatography (MEKC) was developed and introduced into the research field of CE by Terabe *et al.* [18] the application area of CE has been remarkably broadened. MEKC has proven to be useful for the analysis of the NSAIDs in different kind of samples [9,14,19]. However, the low concentration sensitivity is one of main problems in MEKC, as in the other modes of CE. To surmount this problem, many off-line, on-line and on-column preconcentration procedures have been applied in MEKC [20]. The main on-column preconcentration strategies which have been applied in MEKC for the analysis of NSAIDs are based on sample stacking [21]. In this case, the preconcentration of analytes is related on the differences in their electrophoretic migrating velocities between the sample solution and the background electrolyte. By using this general strategy, NSAIDs have been determined in mineral waters by stacking with reverse migration micelles (SRMM), stacking with reverse migrating micelles–anion selective exhaustive injection (SRMM-ASEI) and field-enhanced sample injection with reverse migration micelles (FESI-RMM)[14] and in saliva by sample stacking (LVSS) with polarity switching, named reverse electrode polarity stacking mode (REPSM) [22].

Another on-column concentration technique widely applied in MEKC is sweeping. It was introduced by Quirino and Terabe in 1999 [23] to describe a new approach for the preconcentration of neutral analytes in MEKC. This method is based on the injection of a large volume of sample devoid of pseudostationary phase (PS) and subsequent picking and accumulation of

the analytes into a narrow zone by the PS that penetrates the sample region.

In most cases, the protocol for sweeping neutral and anionic analytes uses an anionic pseudostationary phase (PS) and suppressed EOF in a low pH BGE provided by phosphoric acid or phosphate buffer (pH 2–3) [24-32]. In general, up to 1500- fold improvement in concentration sensitivity can be achieved in the determination of different analytes. The most commonly used anionic pseudostationary phase has been SDS and this has been used for the analysis of Chinese medicinal preparation [24], abused drugs in urine samples [25,26], herbicides in water samples [27] and pesticides in drinking water samples [28]. Cationic surfactants, such as dodecyltrimethylammonium bromide (DTAB), cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTAC) and tetradecyltrimethylammonium bromide (TTAB), have been used for different applications such as the analysis of flunitrazepan and its metabolites [29]. Nonionic surfactants, like Brij 35, have also been used for the determination of phenol derivates [30]. Other pseudostationary phases used are mixed micelles [31] and microemulsions [32].

The resulting approach to combine sweeping and stacking protocols has also been used for determining a group of NSAIDs in water samples [9].

The main aim of this work has been to develop a simple and sensitive on-column sweeping method for the preconcentration and separation of five NSAIDs (ibuprofen, fenoprofen, naproxen, diclofenac sodium, and ketoprofen). The use of a BGE in the acidic range to suppress the EOF has been proposed. Under these conditions, NSAIDs are uncharged, so they could be preconcentrated by sweeping using SDS as a pseudostationary phase (PS). The sample matrix composition and the sample injection time were optimized to obtain the optimum sensitivity enhancement factors.

Finally, river water samples fortified with the NSAIDs compounds were analized to evaluate the applicability of the sweeping methodlogy without any previous pretreatment of the sample.

2. MATERIAL AND METHODS

2.1. Standards and reagents

Standards of the five NSAIDs (ibuprofen, fenoprofen, naproxen, diclofenac sodium, and ketoprofen), acid orthophosphoric acid 85 % (H_3PO_4), sodium dodecyl sulfate (SDS) and dihydrogenphosphate (NaH_2PO_4), and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (98 %) was from Panreac (Barcelona, Spain). Methanol and acetonitrile were from SDS (Peypin,

France). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A stock standard solution of 1000 mg/L of each NSAID was prepared in methanol. A mixed standard solution of the five NSAIDs was prepared weekly at a concentration of 100 mg/L in methanol. The working standard solutions were prepared daily by diluting the stock standard solutions in an appropriate volume of phosphate buffer. All solutions were stored in a dark glass flask at 4 °C. Stock solutions of 250 mM SDS and 100 mM of NaH_2PO_4 were prepared every week in purified water. BGE was prepared (each day) by dilution of the SDS stock solution in an appropriate volume of phosphate buffer. Finally, the pH was adjusted at 2.5 with diluted H_3PO_4. All buffers and working solutions were sonicated and filtered through a 0.22 µm nylon syringe filter (Tracer, Teknokroma, Barcelona, Spain) before used.

2.2. Instrumentation

CE analyses were performed on a Hewlett-Packard 3DCE instrument (Agilent Technologies, Waldbronn, Germany) system equipped with a UV diode-array detector (DAD) operating at 214 nm. The fused-silica capillaries (Polymicro technologies, Phoenix, AZ, USA) had a total length of 60 cm (51.5 cm effective length) and 75 µm I.D. The capillary temperature was set at 25 °C.

For conductivity measurements, a Lab conductivity meter Basic 30+ (Crison, Barcelona, Spain) was used, and for pH measurements a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used.

New capillaries were flushed with 1 M NaOH at 30 °C for 20 min, with Milli-Q water for 20 min until the temperature reached 25 °C and with the acidic BGE solution for 30 min. Each day the capillaries were equilibrated by rinsing with 0.1 M NaOH for 5 min, then with Milli-Q water for 10 min and finally with the acidic BGE solution for 15 min. After each analysis the capillary was rinsed for 3 min with Milli-Q water, 2 min with methanol and 2 min with Milli-Q water. Between analysis the capillary was rinsed for 2 min with 0.1 M NaOH , then with Milli-Q water for 3 min and finally with the acidic BGE solution for 5 min to ensure a good reproducibility.

2.3. Sweeping-MEKC

In the sweeping-MEKC procedure, the capillary was initially filled with BGE (75 mM SDS in 25 mM NaH_2PO_4 with 40 % ACN at pH 2.5). Then, the sample prepared in sodium dihidrogen-phosphate buffer (75 mM NaH_2PO_4) at pH 2.5 (devoid of micelles), having a con-ductivity similar of the BGE (~6.0

mS/cm), was injected hydrodynamically at 50 mbar for 350 s. Then the capillary inlet was placed into the BGE solution and a negative voltage (-26 kV) was applied to begin the sweeping process. The anionic SDS micelles were moved from the inlet to the outlet, picking and accumulating the analytes by sweeping and causing the subsequent separation by MEKC.

2.4. Water Samples

River water samples were collected from Ebro River, Catalonia (Spain). All samples were collected by using precleaned amber glass bottles and acidified to pH 2.5 with HCl, filtered through a 0.22 µm nylon syringe and stored at 4 °C until analysis. Before their analysis, 100 ml of river water sample was diluted 1:5 with a solution of 95 mM of sodium dihidrogenphosphate pH 2.5, in such a way that the conductivity of the sample is equal to the BGE conductivity.

3. RESULTS AND DISCUSSION

3.1. Separation of NSAIDs using an acidic micellar BGE solution

An acidic micellar BGE solution was chosen to separate the drugs and reduce the EOF in order to apply an on-column preconcentration technique without polarity switching. The experiments were carried out with 25 mM sodium dihydrogenphosphate, as the aqueous buffer medium, and 75 mM SDS, as the pseudostationary phase, containing 40 % acetonitrile at pH 2.5 [14]. The separation process was performed at a constant negative voltage, -26 kV, this ensured the negatively charged micelles migrated in the direction of the detector.

3.2. Optimization of the sweeping conditions

Sweeping is based on the picking and the accumulation of analyte molecules by the pseudostationary phase (PS) that penetrates the sample zone during voltage application. To obtain the optimum preconcentration results, the effects of two parameters in the sweeping-MEKC method were studied: the sample matrix conductivity and the sample injection time. Our attempts to optimize each of these conditions are described in detail below.

3.2.1. Effect of the sample matrix conductivity on the stacking efficiency

The ratio of conductivity values of the sample matrix and the BGE affects the distribution of electric field strength across the capillary and influences the sweeping results [33,34]. To examine the influence of sample conductivity on the sweeping effect, the conductivity

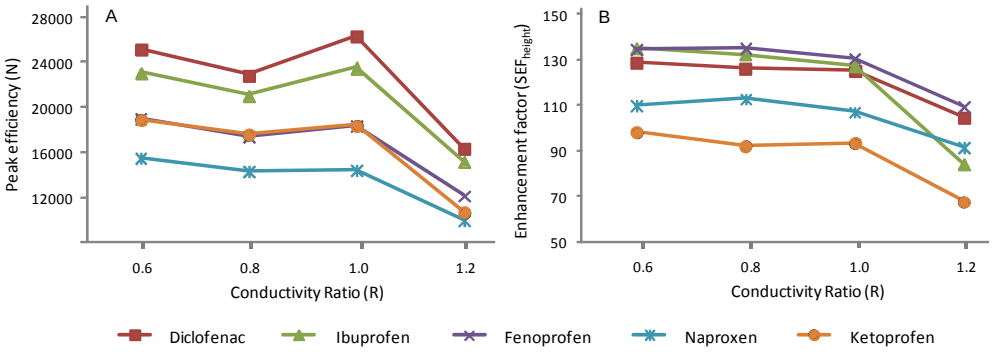

Figure 1. Effect of sample matrix conductivity on: (A) peak efficiency; (B) sensitivity enhancement factor (SEF_{height}). Conditions: BGE, 75 mM SDS in 25 mM NaH_2PO_4 (pH 2.5) containing 40% acetonitrile; sample concentration, 0.5 µg/mL; sample injection time, 50 mbar for 300 s; separation voltage, -26 kV. Conductivity ratio: 0.6, 0.8, 1.0, and 1.2. In all the cases the experiments were performed by triplicate.

ratio of the sample and the BGE was investigated in terms of peak height sensitivity enhancement factor (SEF_{height}) and peak efficiency (N). To carry out this study the analytes were dissolved in an aqueous solution containing 25, 50, 75 and 100 mM NaH_2PO_4 at pH 2.5, with conductivity ratios of 0.6, 0.80, 1.0 and 1.2, respectively.

Figure 1A shows the effect of conductivity ratio on the peak efficiency (N). The best efficiency is obtained when the conductivity ratio is 1, in other words, when the sample conductivity is equal to the BGE conductivity. The constant electric field inside the capillary involves that the concentration of the micelles that will enter the sample zone will be similar than that in the BGE [23]. However, a decrease in the efficiency is observed when the conductivity ratio is lower or higher than 1. When the conductivity of the sample matrix is lower than the conductivity of the BGE, the concentration of the micelle that will enter the sample zone upon application of voltage will be lower than that in the BGE [34,35] involving a loss of efficiency. When the conductivity of the sample matrix is higher than the conductivity of the BGE, the sweeping preconcentration involves three important processes [34]. First, stacking of the micelles at the cathodic interface between the sample solution (S) and the BGE zones is identified. This is then followed by the sweeping of analyte molecules by the stacked micelles that enter the S zone. Finally, the destacking of the stacked micelles at the anodic interface between the S and BGE zones occurs. As it is observed, the efficiency decreases, due the destacking process occurred in the last step. In Figure 1B

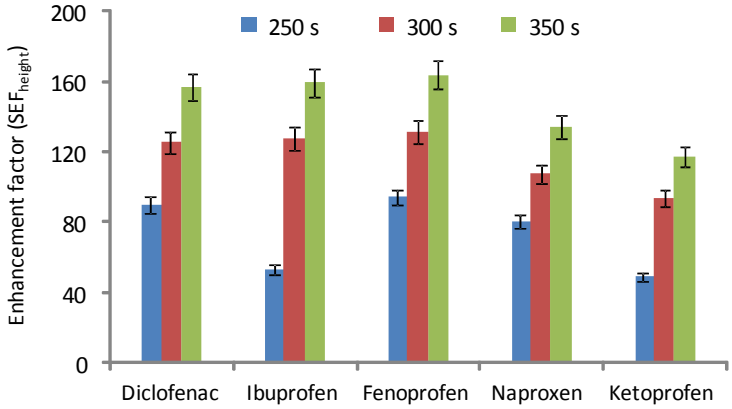

Figure 2. Effect of the hydrodynamic injection time on the peak height sensitivity enhancement factors (SEF_{height}). Conditions: BGE, 75 mM SDS in 25 mM NaH_2PO_4 (pH 2.5) containing 40% acetonitrile; sample concentration, 0.5 µg/mL in 75 mM NaH_2PO_4 (pH 2.5); separation voltage, -26 kV. Sample injection time: 250, 300, and 350 s. In all the cases the experiments were performed by triplicate.

the relationship between the SEF_{height} and the conductivity ratio is represented. It can be observed that the focusing effect of sweeping with an equal or lower sample conductivity compared to the BGE is roughly the same, however, when the conductivity of the sample is higher than the conductivity of the BGE, there is a slight decrease in the peak height sensitivity enhancement factors. As it has been mentioned previously, a destacking process is observed when conductivity ratios are greater than 1, and this affects the efficiency as well as the SEF_{height}.

To obtain the best SEF in less time and with more efficiency, the conductivity of the sample was adjusted to the conductivity of the BGE. In this way, the samples were prepared in a concentration about 75 mM of sodium dihydrogenphosphate, that match the conductivity of the BGE.

3.2.2. Effect of sample injection time on the stacking efficiency

Injection time affects the amount of sample introduced in the CE and hence has a direct influence on sample preconcentration effects. For sweeping technique, the resulting length of the injected analyte zone (l_{sweep}) is theoretically narrowed by a factor equal to $1/(1 + k)$ according to the Eq. 1, where k is the retention factor and (l_{inj}) the length of the initial zone:

$$l_{sweep} = l_{inj} \frac{1}{1+k} \qquad (1)$$

The sample solution was injected at 50 mbar for 250, 300 and 350 s into the column, corresponding to the injected length of 35.4, 42.5, and 49.5 cm, respectively, calculated according to the Poiseuille law. The results are shown in Figure 2, and as can be observed an increase of the peak height sensitivity enhancement factor was obtained with the increase of the injection time. However, a decrease of the resolution and the efficiency were also obtained. When the injection time was 350 s, the analytes provided a properly peak separation and efficiency with the highest stacking sensitivity. In our case we have relatively high current values, and this can lead to a irreproducibility in the electroosmotic flow especially if the current increases as it happens with injection time higher than 350 s.

Comparison of the electropherograms obtained from the optimum sweeping-MEKC method and the normal-MEKC method are shown in Figure 3. Under the above optimized experimental conditions, around 163- and 401- fold improvements in peak height and peak area, respectively, were achieved.

3.3. Validation of the analytical method

Table 1 lists the linearity, calibration curve equations, limits of detection (LODs), repeatability and reproducibility (in terms of % RSD) of the peak height and corrected peak area, and sensitivity enhancement factors (SEF) under the optimal sweeping-MEKC conditions. Calibration graphs for the studied NSAIDs were linear in the tested range and the limits of detection (LODs, S/N = 3) for the five analytes ranged from 1.2 to 2.5 ng/mL. The repeatability (intra-day) was assessed as the relative standard deviation (% RSDs) of the peak area and of the peak height of replicate experiments (n=5) at two different concentrations: 20 and 100 ng/mL. The day-to-day reproducibility, expressed also by relative standard deviation (%RSDs), was calculated by performing three replicate experiments at two different concentrations, 20 and 100 ng/mL, in four different days (n=4). The sensitivity enhancement factor for each compound was within the range of 298-401 for SEF_{area} and 113-163 for SEF_{height}. The low values of SEF_{height} in comparison with the corresponding values for SEF_{area} can be attributed to the loss of efficiency and resolution when sweeping is applied. Loss of efficiency involves that the area increases at a higher rate than that of the peak height. The SEF achieved with this sweeping technique for the NSAIDs was in agreement with results reported in the literature for other kind of compounds [24,26,28].

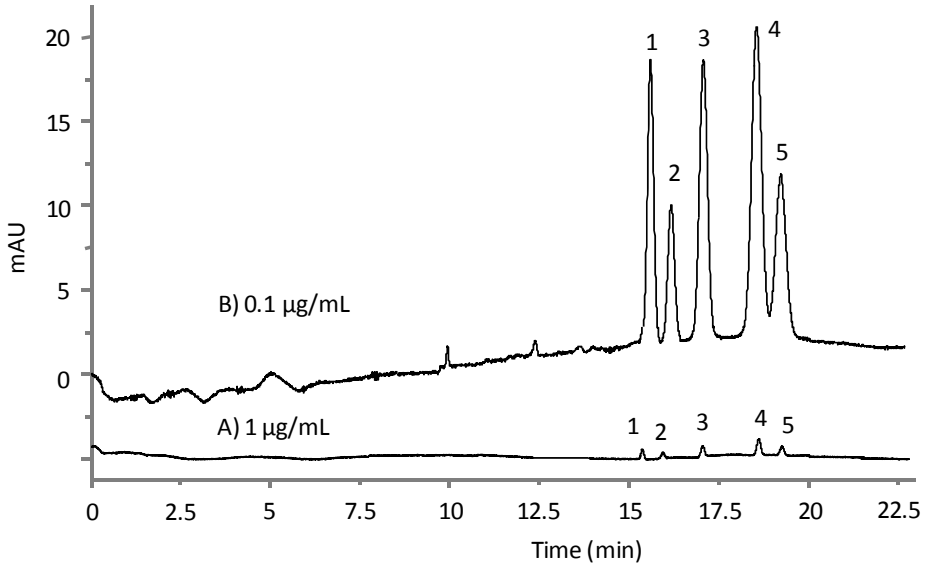

Figure 3. Comparison between the normal MEKC and sweeping-MEKC methods. (A) Normal MEKC conditions: BGE, 75 mM SDS in 25 mM NaH_2PO_4 (pH 2.5) containing 40% acetonitrile; sample concentration, 1.0 µg/mL in water; sample injection, 50 mbar for 5 s; separation voltage, -26 kV (B) Sweeping-MEKC conditions: BGE, 75 mM SDS in 25 mM NaH_2PO_4 (pH 2.5) containing 40% acetonitrile; sample concentration, 0.1 µg/mL in 75 mM NaH_2PO_4 (pH 2.5); sample injection, 50 mbar for 350 s; separation voltage, -26 kV. Peak designation: (1) diclofenac; (2) ibuprofen; (3) fenoprofen; (4) naproxen; (5) ketoprofen.

3.4. Application

To test the applicability of the developed method based on sweeping MEKC, river water samples were analyzed. First, we carried out a direct injection of the river water spiked with 100 ng/mL of each of the drugs. The resulting separation remained poor and the sweeping effect was almost negligible, since the sample composition has an important effect over this process. For that reason a dilution of the sample was necessary before the injection. To find the most suitable dilution factor, we performed different experiments. River water samples were diluted 1:2, 1:5 and 1:10 with 150, 95, and 85 mM sodium dihidrogenphosphate pH 2.5, respectively, to assure to match the conductivity values of the sample matrix and the BGE, and spiked with 100 ng/ mL of each of the drugs. The dilution 1:5 of the river water was found to be the most suitable, giving a good separation and the best sweeping effect. Figure 4Aa shows the electropherogram obtained from the injection of the blank of river water diluted 1:5 to verify the absence of peaks at the same migration time of the NSAIDs, and Figure 4Ab shows the

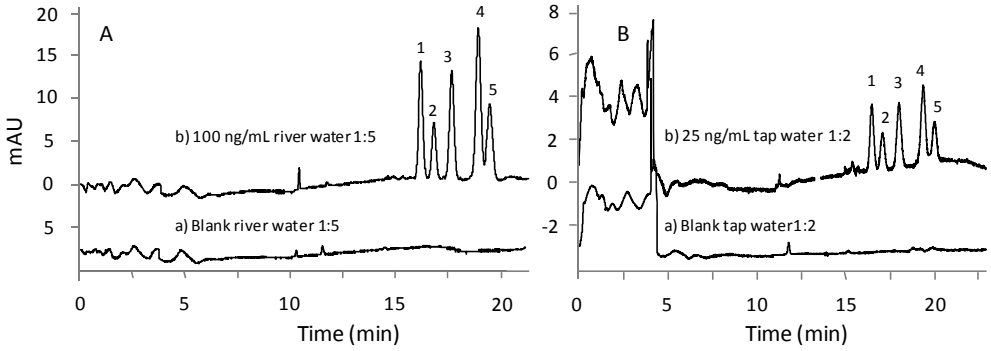

Figure 4. Electropherogram obtained from direct injection of (Aa) Blank and (Ab) 100 ng/mL spiked river water diluted 1:5 with Milli-Q water, having the same conductivity than the BGE (pH 2.5) and (Ba) Blank and (Bb) 25 ng/mL spiked of tap water diluted 1:2 with Milli-Q water, having the same conductivity than the BGE. For other experimental conditions, see Fig 3B.

electropherogram obtained from the direct injection of river water diluted 1:5 and spiked with 100 ng/mL of NSAIDs.

Table 1 shows the linearity, calibration curve equations, limits of detection (LODs), and repeatability and reproducibility of the peak height and corrected peak area (in terms of %RSD) when river water samples diluted 1:5 with 95 mM of sodium dihidrogenphosphate at pH 2.5, spiked with the NSAIDs at different concentrations were analysed under the optimal sweeping-MEKC conditions. As it can be observed, the calibration graphs for the studied NSAIDs were linear in the tested range and the limits of detection (LODs, S/N = 3) for the five analytes ranged from 6.5 to 14.6 ng/mL. The repeatability (intra-day) was assessed as the relative relative standard deviation (% RSDs) of the peak area and peak height of replicate experiments (n=5) at two different concentrations: 100 and 500 ng/mL. The day-to-day reproducibility, expressed also by relative standard deviation (% RSDs), was calculated by performing three replicate experiments at two different concentrations, 20 and 100 ng/mL, in four different days (n=4).

The proposed preconcentration strategy was also tested for tap water. In this case, a 1:2 dilution with 150 mM of sodium dihidrogenphosphate at pH 2.5 was found to be suitable. Figure 4Ba shows the electropherogram obtained from the injection of the blank of tap water diluted 1:2 to verify the absence of peaks at the same migration time of the NSAIDs, and Figure 4Bb shows the electropherogram obtained from the direct injection of tap water diluted 1:2 and spiked with 25 ng/mL.

The results obtained for the group of NSAIDs in water samples in this work were compared to those obtained by

Table 1. Linearity, calibration curves, LODs, repeatability (%RSD) and reproducibility (%RSD) of the peak height and corrected peak area, and sensitivity enhancement factors (SEF) for standard samples and for river water samples.

Compounds	Linearity (ng/mL)	Calibration graphs			Repeatability (% RSD) (n=5) (20 ng/mL)		Repeatability (% RSD) (n=5) (100 ng/mL)		Reproducibility (% RSD) (n=5) (20 ng/mL)		Reproducibility (% RSD) (n=5) (100 ng/mL)		SEF [a,b]	
		Calibration curve	R^2	LOD (ng/mL)	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area	SEF_{area}	SEF_{height}
Standard samples														
Diclofenac sodium	5-500	y= 2.86x - 7.29	0.998	1.4	2.5	4.6	3.3	5.1	3.3	5.1	3.9	6.6	346	156
Ibuprofen	10-500	y= 1.38x + 4.42	0.998	2.5	1.8	4.5	2.0	7.3	2.0	7.3	4.8	5.3	338	159
Fenoprofen	5-500	y= 4.13x + 2.30	0.998	1.2	2.7	3.8	4.3	4.2	4.3	4.2	7.3	6.6	401	163
Naproxen	3-500	y= 6.29x - 4.28	0.999	1.2	2.3	5.2	4.4	5.8	4.4	5.8	3.5	7.8	357	134
Ketoprofen	10-500	y= 2.50x + 28.6	0.998	2.3	4.5	7.2	11.4	9.0	11.4	9.0	13.8	11.2	298	113
River water samples														
Diclofenac sodium	25-1250	y= 1.43x + 3.93	0.997	8.4	3.1	5.1	3.3	5.8	4.2	8.7	4.7	11.1	-	-
Ibuprofen	50-1250	y= 0.78x - 0.12	0.997	14.6	2.7	3.4	4.8	5.1	5.3	4.8	7.0	3.8	-	-
Fenoprofen	25-1250	y= 1.58x + 12.1	0.995	7.8	3.4	4.1	5.3	6.2	6.6	3.7	8.3	3.2	-	-
Naproxen	25-1250	y= 2.23x + 19.2	0.996	6.5	2.8	5.5	4.5	5.9	5.4	3.8	4.0	2.3	-	-
Ketoprofen	50-1250	y= 1.15x - 16.3	0.994	13	4.4	6.7	7.7	8.2	5.5	3.2	4.9	1.9	-	-

[a] Sensitivity enhancement factor for standard samples: SEF_{area} = dilution factor × ($area_{sweeping}/area_{normal\ hydrodynamic\ injection}$);
SEF_{Height} = dilution factor × ($height_{sweeping}/height_{normal\ hydrodynamic\ injection}$).

[b] Sensitivity enhancement factors for river water samples were not evaluated.

different preconcentration approaches based on sample stacking techniques, EKS, in-line SPE-CE, ASEI-sweeping-MEKC or by CZE-MS. In those studies, NSAIDs were detected at a concentration range from 0.05 to 1.6 ng/mL by MEKC with SRMM, SRMM-ASEI and FESI-RMM [14], and from 0.1 to 0.250 ng/mL by MEEKC with REPSM when mineral water samples were analysed [13], 0.1 ng/mL by CZE-MS for surface water samples [36], and 0.01 ng/mL by an in-line SPE-CE for tap water samples [15.] It is important to remark, that in all these previous studies, a SPE was performed off-line before the electrophoretic method and in general, the preconcentration factors for this off-line pretreatment were between 100-fold and 1000-fold. Taking into account those preconcentration factors, we can conclude that the LODs achieved in our case for river waters, ranging from 6.5 to 14.6 ng/mL, are comparable to the ones reported previously in the bibliography which use a more exhaustive pretreatment of the sample. Recently, some authors use EKS as the electrophoretic preconcentration technique for NSAIDs. In particular, we can mention the work of Dawod *et al.* [11] and Botello *et al.* [10] in which the authors obtained LODs about 2 ng/mL which are similar to our results. However, more recently Dawod *et al.* [12] could detect lower concentrations for these compounds when they applied this strategy with and additional negative hydrodynamic pressure during sample injection, that allows the introduction of a higher sample volume. Another strategy reported recently for the preconcentration of NSAIDs was ASEI-sweeping which allowed detection limits for river water samples between 29 and 58 ng/mL after a simple dilution of the sample [9].

The main advantages of the developed method are related to its simplicity, only a dilution and the conductivity adjustment of the sample are required prior to its analysis, and also to a low influence of the sample matrix due to the hydrodynamic sample injection, in comparison with preconcentration techniques that involve electrokinetic sample injection, as EKS [10-12] or ASEI-sweeping [9]. On the other hand, one of the main drawbacks of sweeping is the limited sample volume that can be injected.

4. CONCLUSIONS

It has been the first time that a sweeping-MEKC method has been developed to preconcentrate and separate a group of NSAIDs, achieving 113-163-folds and 298-401-folds improvements in peak height and peak area respectively, and LODs ranging between 1.2 and 2.5 ng/mL. The paper

also demonstrated the potential of the sweeping methodology for the analysis of environmental waters, through the analysis of diluted river and tap water samples fortified with the NSAIDs. The LODs obtained ranged between 6.5 and 14.6 ng/mL. Through the obtained results we can verify the potential of sweeping as a preconcentration technique for NSAIDs in MEKC. Further studies to decrease the limits of detection will be focused on the combination of the developed methodology with a stacking protocol or a sample pretreatment, such as solid-phase extraction.

Acknowledgements

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3.1.2. On-column preconcentration of anti-inflammatory drugs in river water by anion-selective exhaustive injection-sweeping-MEKC

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PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
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ON-COLUMN PRECONCENTRATION OF ANTI-INFLAMMATORY DRUGS IN RIVER WATER BY ANION-SELECTIVE EXHAUSTIVE INJECTION-SWEEPING-MEKC

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Abstract

A high-sensitivity on-column preconcentration method, anion selective exhaustive injection (ASEI)-sweeping in micellar electrokinetic chromatography (MEKC) has been developed for the analysis of non-steroidal anti-inflammatory drugs (NSAIDs): diclofenac sodium, ibuprofen, fenoprofen, naproxen and ketoprofen in water samples. To achieve the best results of the ASEI-sweeping-MEKC method, conditions which affected preconcentration were examined, including the sodium dodecyl sulfate (SDS) concentration, composition of the sample matrix, composition and injection length of the water plug, the concentration and the injection length of the high-conductivity buffer (HCB), and finally the sample injection time. Under the optimum stacking conditions the method was validated for the determination of the studied NSAIDs in river water samples with limits of detection (LODs) ranging between 29 and 58 ng mL^{-1}, and without any previous sample pretreatment.

Keywords: Anion-selective exhaustive injection (ASEI), micellar electrokinetic capillary chromatography (MEKC), non-steroidal anti-inflammatory drugs (NSAIDs), river water, sweeping.

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the group of analgesics and anti-inflammatory drugs most commonly used in human and veterinary medicine and often found in the aquatic environment. Owing to their hydrophilicity and stability, NSAIDs tend to remain in the aqueous phase and are not totally eliminated by the sewage treatment plants and as a consequence they are frequently detected in water. The analysis of these compounds in the aquatic environment involve a laborious task, associated with the complexity of the matrices and their low concentration

levels in water. In this sense, very sensitive and selective analytical methodologies have received considerable attention in recent years.

The main techniques used for the analysis of such compounds are liquid chromatography-tandem mass spectroscopy (LC-MS-MS) [1,2] and gas chromatography-mass spectrometry (GC-MS) [3,4]. CE combined with different preconcentration techniques has also been applied to determine these compounds in water samples [5-8].

Since micellar electrokinetic capillary chromatography (MEKC) was developed and introduced into the research field of CE by Terabe *et al.* [9] the application area of CE has been remarkably broadened. MEKC proved to be useful for the analysis of NSAIDs in different kinds of samples [7]. However, the low concentration sensitivity is one of main problems in MEKC, as in the other modes of CE. To surmount this problem, many off-line, on-line and on-column preconcentration procedures have been applied in MEKC [10]. The main on-column preconcentration strategies which have been applied in MEKC for the analysis of NSAIDs are based on sample stacking [11]. In this case, the preconcentration of analytes is related to the differences in their electrophoretic migrating velocities between the sample solution and the background electrolyte (BGE). Using this general strategy, NSAIDs have been determined in mineral water by stacking with reverse migration micelles (SRMM), stacking with reverse migrating micelles-anion selective exhaustive injection (SRMM-ASEI) and field-enhanced sample injection (FESI) with reverse migration micelles (FESI-RMM) [7] and in saliva by sample stacking (LVSS) with polarity switching, named reverse electrode polarity stacking mode (REPSM) [12].

Another on-column concentration technique widely applied in MEKC is sweeping [13]. In sweeping, neutral and charged analytes are picked up by a pseudostationary phase that penetrates the sample zone, and the affinity of this phase for analytes determines the concentrating ability.

Because of the nature of sweeping, this strategy can be used in conjunction with other stacking methods to sweep zones that have already been stacked, and the result is a higher enhancement in the sensitivity. This area was pioneered by Quirino and Terabe [14] who combined FESI with sweeping and they called this strategy selective exhaustive injection-sweeping (SEI-sweeping). They could increase the sensitivity by almost 100,000-fold using this combination. The electrokinetic injection is selective for either cationic or anionic analytes and in this way there are two different methodologies depending on the characteristics of the

analytes: cation selective exhaustive injectionsweeping (CSEI-sweeping) or anion selective exhaustive injection-sweeping (ASEI-sweeping).

The first studies reported in the bibliography in which they use this combination were performed with CSEI-sweeping [15-17]. Kim *et al.* [18] introduced ASEI-sweeping using a polyacrilamide (PAA)-coated capillary to suppress the EOF and cationic micelles as carriers for the anions. Some aromatic carboxylic acids, dansyl amino acids, and naphthalenedisulfonic acids were concentrated by ASEI-sweeping-MEKC. Under the optimized conditions, about 1,000 to 6,000-fold increases in detection sensitivity were obtained. Later, Zhu *et al.* [19] analyzed six flavonoids in Chinese herbal medicine, and an increase in sensitivity of 13 to 31-fold was found in terms of peak height, and the limits of detection (LODs) achieved for standards ranged between 2.4 and 12.8 ng mL^{-1}. The combination of ASEI-sweeping and microemulsion electrokinetic capillary chromatography (MEEKC) has also been explored to improve the sensitivity in CE [20-22].

The main aim of this work has been to examine the possibility of using ASEI-sweeping method as an on-column preconcentration technique to determine NSAIDs in river water samples by MEKC. The optimization of different parameters such as the SDS concentration, the sample matrix composition, the water plug, the high-conductivity buffer (HCB) plug, and the injection time of the sample, was performed in order to obtain the optimum enhancement sensitivity fac-tor. To the best of our knowledge, this is the first study using ASEI-sweeping as an oncolumn preconcentration technique in combination with MEKC to determine NSAIDs in river water samples.

2. MATERIAL AND METHODS

2.1. Standards and reagents

Standards of the five NSAIDs (ibuprofen, fenoprofen, naproxen, diclofenac sodium, and ketoprofen), ortho-phosphoric acid 85 % (H_3PO_4), sodium tetraborate anhydrous ($Na_2B_4O_7$), sodium dodecyl sulfate (SDS) and sodium dihydrogen-phosphate (NaH_2PO_4) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (98%) was from Panreac (Barcelona, Spain). Methanol and acetonitrile were from SDS (Peypin, France). Ultrapure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A stock standard solution of 1,000 mg L^{-1} of each NSAID was prepared in methanol. A mixed standard solution of the five NSAIDs was prepared weekly at

a concentration of 100 mg L^{-1} in methanol. The working standard solutions were prepared daily by diluting the stock standard solutions in an appropriate volume of Milli-Q water and adjusted to pH 9 with 0.1 M NaOH. All solutions were stored in a dark-glass flask at 4 °C. Stock solutions of 250 mM SDS and 100 mM of NaH_2PO_4 (HCB) were prepared every week in purified water. The background electrolyte (BGE) was composed by 50 mM SDS in 20 mM sodium dihydrogenphosphate with 30 % acetonitrile at pH 2.5 adjusted with diluted H_3PO_4. All buffers and working solutions were sonicated and filtered through a 0.22 µm Nylon syringe filter (Tracer, Teknokroma, Barcelona, Spain) before used.

2.2. Instrumentation

CE analyses were performed on a Hewlett-Packard 3DCE instrument (Agilent Technologies, Waldbronn, Germany) system equipped with a UV diode-array detector (DAD) operating at 214 nm. Separations were performed in a 60 cm total length (51.5 cm to the detector) of 50 µm I.D uncoated fused-silica capillary (Polymicro technologies, Phoenix, AZ, USA). The capillary temperature was set at 25 °C. The separation voltage was -27 kV.
New capillaries were flushed with a 1 M NaOH solution at 30 °C for 20 min, with Milli-Q water for 20 min until the temperature reached 25 °C and with the acidic BGE solution for 30 min. Each day the capillaries were equilibrated by rinsing with 0.1 M NaOH for 5 min, then with Milli-Q water for 10 min and finally with the acidic BGE solution for 15 min. After each analysis the capillary was rinsed for 3 min with Milli-Q water, 2 min with methanol and 2 min with Milli-Q water. Between analysis the capillary was rinsed for 2 min with the 0.1 M NaOH solution, then with Milli-Q water for 3 min and finally with the acidic BGE solution for 5 min to ensure a good reproducibility.

2.3. Water Samples

River water samples were collected from Ebro River, Catalonia (Spain). All samples were collected by using pre-cleaned amber glass bottles and acidified to pH 3 with HCl, filtered through a 0.22 µm nylon filter (Whatman, Maidstone, UK) and stored at 4 °C until analysis. River water samples were diluted 1:10 with Milli-Q water and then pH was adjusted to 9.

2.4. ASEI-sweeping-MEKC

The different steps of the ASEI-sweeping MEKC method developed in this study are shown in Figure. 1a–f. First, (a) the capillary is filled with an acidic micellar BGE solution (pH 2.5) in order to suppress the electroosmotic

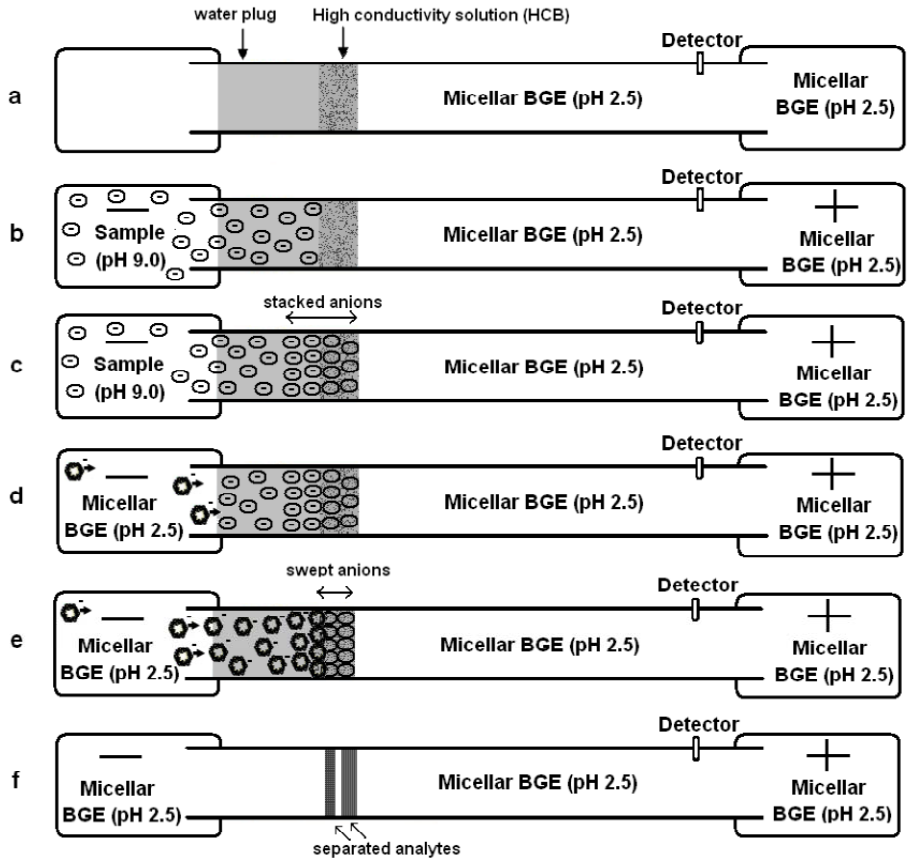

Figure 1. Schematic diagram of the ASEI-sweeping MEKC process. (a) A capillary was conditioned with a BGE solution of pH 2.5, a short HCB and a short water plug were then introduced hydrodynamically, 50 mbar at 15 and 60 s, respectively. (b) Electrokinetic injection for 50 s at negative polarity (-20 kV) of the NSAIDs prepared in Milli-Q water at pH 9. (c) Once the sample anions reach the boundary between the water plug and the acidic HCB, anionic analytes are neutralized, and are stacked at the interface. (d) BGE solution was placed at the inlet end of the capillary followed by the application of voltage at negative polarity (-27 kV). (e) The micelles sweeped the stacked analytes into a narrower band, and (f) subsequent separation was achieved under MEKC mode.

flow. Subsequently, a plug of HCB composed of 100 mM NaH_2PO_4 at pH 2.5 is introduced hydrodynamically at 50 mbar for 15 s, followed by a water plug [Milli-Q water: ACN (70:30 v/v)] at 50 mbar for 60 s; (b) a negative voltage (-20 kV) is applied for 50 s, and the NSAIDs prepared in a Milli-Q water solution at pH 9 are electrokinetically injected into the capillary; (c) once the sample anions reach the boundary between the water plug and the acidic HCB, anionic analytes are neutralized, and they are stacked at the interface; (d) injection is stopped and the BGE is placed in the inlet vial and a negative

voltage of -27 kV is applied, allowing the entry of micelles from the cathodic vial into the capillary; (e) the micelles sweept the stacked analytes into a narrow band and; (f) subsequent separation is achieved under MEKC mode.

3. RESULTS AND DISCUSSION

3.1. Separation of NSAIDs using an acidic micellar BGE solution

The MEKC separation was performed with a BGE composed by 50 mM SDS in 20 mM sodium dihydrogenphosphate at pH 2.5 with 30 % acetonitrile [7]. With this acidic micellar BGE solution the EOF was effectively suppressed. The separation process was performed at a constant negative voltage; this ensured the negatively charged micelles migrated in the direction of the detector. Under these conditions, the analytes were separated only by their partition between the micelle and the aqueous phase. The NSAIDs were uncharged at the studied pH value since the pKa values of the compounds ranged from 4.15 to 4.40. The analysis time for the five NSAIDs was < 15 min.

3.2. Optimization of the ASEI-sweeping conditions

In an attempt to improve the detection sensitivity, ASEI-sweeping-MEKC was chosen as the on-column preconcentration procedure and for the subsequent separation of the NSAIDs in water samples. ASEI-sweeping-MEKC is based on the combination of sample stacking and sweeping, introducing the sample electrokinetically. To obtain the largest increase in sensitivity, the following parameters were consecutively investigated: composition of the sample matrix, SDS concentration, the concentration and the injection length of the HCB, composition and injection length of the water plug and finally sample injection time and voltage. Our attempts to optimize each of these conditions are described in detail below.

3.2.1. Optimization of the sample matrix composition

The properties of the sample matrix, including its acidity and conductivity, have significant impact on the sensitivity of ASEI-sweeping [23,24]. The influence of sample pH on NSAIDs stacking was first examined, since the amount of analytes that is introduced during the ASEI step depends on the dissociation degree of analytes in the sample matrix [20].

To carry out this study, before the injection of the sample a plug of Milli-Q water was introduced hydrodynamically for 40 s at 50 mbar. Previous reports have shown that the introduction of this water plug provides a

higher electric field at the tip of the capillary, which will facilitate the entrance of the anions in the capillary, so this will improve the sample stacking procedure [20,25,26].

For the sample pH study, the analytes were prepared in Milli-Q water at two different pH values. In the first case the sample pH was not adjusted (pH of the sample was about 5.5) and in the second case we adjusted it at a value of 9 with NaOH. Then, the electrokinetic injection of the sample was performed for 50 s at -20 kV. The electropherograms of the five NSAIDs are shown in Figure 2. As can be observed, the analytes were not successfully injected and stacked when they were prepared in Milli-Q water without a pH adjustment, as can be observed through Figure 2a. However, when the pH was adjusted at a value of 9, all analytes were successfully injected and stacked (Figure 2b). The pKa values of the compounds ranged from 4.15 to 4.40, so depending on the sample pH, the compounds will be dissociated, not dissociated or partially dissociated. When the pH was not adjusted the five NSAIDs were partially dissociated and consequently, these analytes were not totally injected into the capillary during the ASEI mode. However, when the sample matrix was at pH 9, the five NSAIDs were totally dissociated and under these conditions, when the electric field was applied, the analytes migrated rapidly to the front edge of the sample through the plug of Milli-Q water. So, a suitable pH adjustment of the sample matrix was needed in order to further increase the stacking effect under the ASEI mode.

In this work we use a combination of two preconcentration techniques, ASEI and sweeping, and the sample conductivity affects in a different way to each one. In ASEI a low conductivity is required to obtain high preconcentration levels, and in sweeping, in general, high preconcentration factors are achieved when the conductivities for the sample matrix and for the BGE are similar. To investigate the effect of preparing samples in different solutions over the preconcentration factors, the analytes were prepared in Milli-Q water (C = 13.8 μS cm^{-1}) (Figure 2b), sodium dihydrogenphosphate 1 mM (C = 188 μS cm^{-1}) (Figure 2c) and sodium borate 1 mM (C = 143 μS cm^{-1}) (Figure 2d), all adjusted at pH 9.0. The electropherograms for each case showed an increase on the response when the sample was prepared in phosphate or borate buffers in comparison with the response obtained for Milli-Q water. However, an important split of the peaks was observed when these solutions were used, and for this reason we choose a sample matrix of Milli-Q water adjusted at pH 9 to prepare the analytes for further studies.

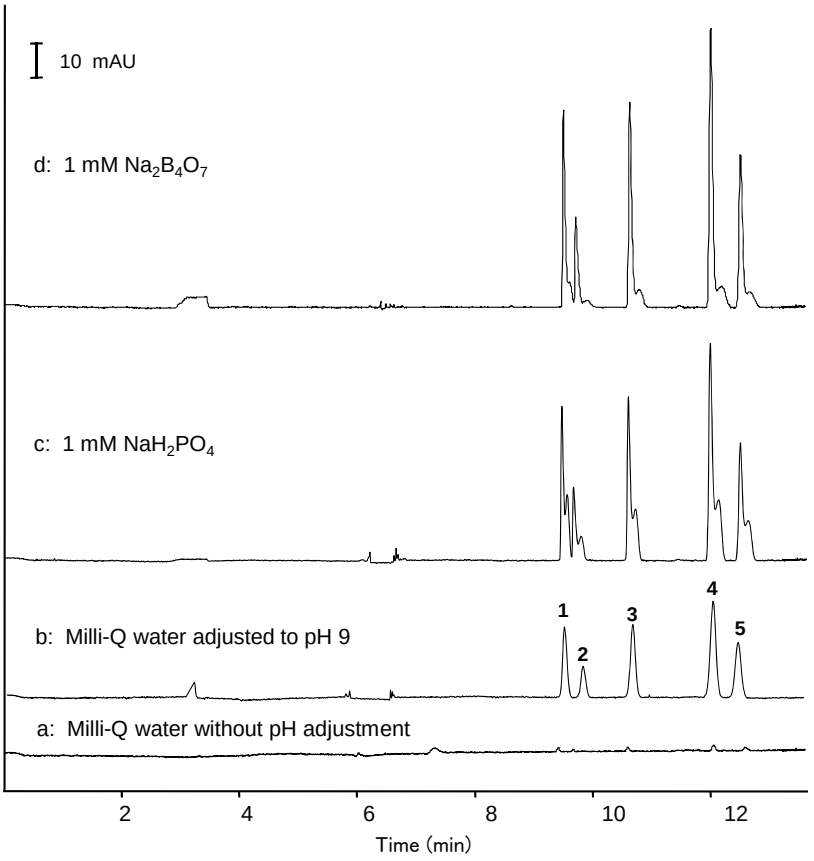

Figure 2. Influence of the sample matrix on the ASEI–sweeping-MEKC method. Conditions: BGE, 50 mM SDS in 20 mM sodium dihydrogenphosphate buffer (pH 2.5) containing 30 % acetonitrile; hydrodynamic injection of Milli-Q water plug for 40 s at 50 mbar; sample concentration, 500 ng mL^{-1}; electrokinetic injection for 50 s at -20 kV; separation voltage, -27 kV. Sample matrix: (a) Milli-Q water without pH adjustment, (b) Milli-Q water adjusted at pH 9, (c) 1 mM NaH_2PO_4 at pH 9, and (d) 1 mM$Na_2B_4O_7$ at pH 9. Peak designation: (1) diclofenac sodium, (2) ibuprofen, (3) fenoprofen, (4) naproxen, (5) ketoprofen.

3.2.2. Optimization of the SDS concentration

In the ASEI-sweeping-MEKC methodology, the concentration of micelles is assumed to be an essential variable as they exert a deep effect on both separation and sweeping concentration [24,26,27]. In an attempt to enhance the enriching sensitivity, the influence of the concentration of SDS was studied in the range of 25-60 mM. As expected, the results indicated that the migration time decreased and the peak heights became higher as the SDS concentration increased from 25 to 50

mM. Concentrations higher than 50 mM did not increase the peak height and a peak broadening was observed. This may be due to the increase of the retention factors of the analytes, and the difference between the electric field strength in sample zone and that in buffer zone with the increase of SDS concentration, which will improve the stacking efficiency [24]. Therefore, considering the separation efficiency and the sensitivity enhancement factor, we concluded that 50 mM was the optimal concentration for SDS.

3.2.3. Optimization of the HCB plug

Previous studies had demonstrated that the stacking efficiency and the peak shapes markedly improved when a HCB zone was injected before the Milli-Q water plug [18,22,24,28], as a result of the different electric field strengths between the Milli-Q water plug and the HCB plug. The HCB was used to form a wall, thereby reducing the anionic analytes' velocities and resulting in the anions stacking in a narrow zone.

The effect of using an HCB plug before the Milli-Q water plug on the stacking and peak efficiency was tested by the injection of three different concentrations of HCB (75, 100, and 150 mM sodium dihydrogenphosphate) for 15 s at 50 mbar, in which the electrokinetic injection of the sample was performed for 50 s at -20 kV. Sodium dihydrogenphosphate was chosen as HCB, since this was the salt used as BGE. The best results were observed when the phosphate concentration was 100 mM. At a concentration of 150 mM, the peak intensity appeared to decrease, the peaks broadened and the baseline noise increased. The length of the HCB plug was evaluated, at a concentration of 100 mM, from 15 to 30 s at 50 mbar and no significant changes in peak intensity and peak efficiency were observed when the length of the HCB injected was increased. So, 15 s were selected as the optimum injection time of the HCB (100 mM NaH_2PO_4).

3.2.4. Optimization of the water plug

As it has been mentioned, the introduction of a water plug before the injection of the sample improves the stacking efficiency as a result of the high electric field created at the tip of the capillary, which will accelerate the anions into the capillary. Therefore, the effect of this plug on the NSAIDs stacking was examined.

The water plug composition has an important effect in the stacking efficiency, as it has been demonstrated previously in the bibliography through the study of the addition of an organic solvent into this plug [19]. As acetonitrile is the organic solvent used in our BGE, it was added into the Milli-Q

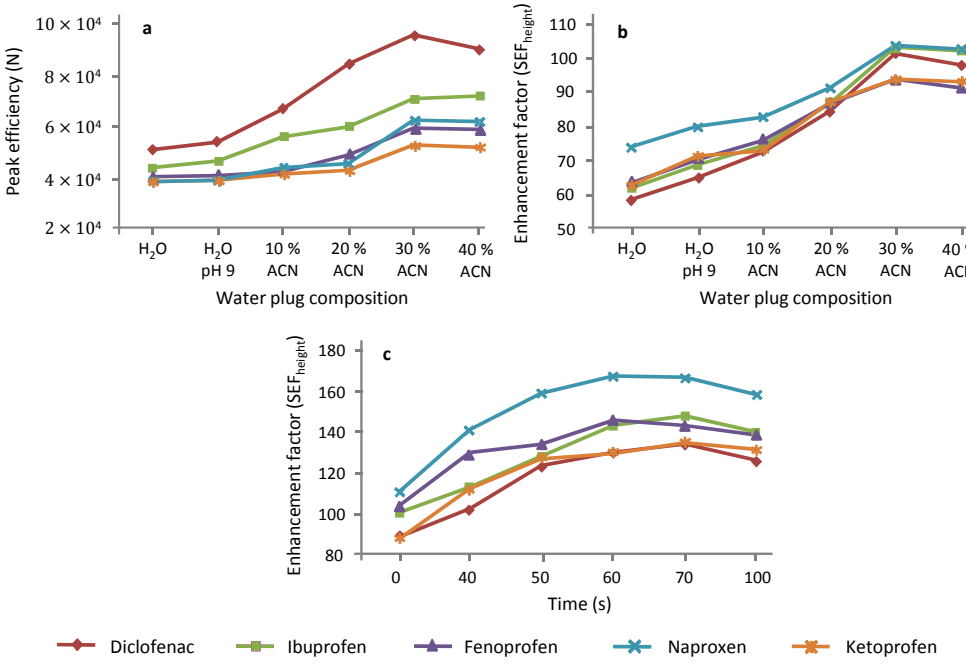

Figure 3. (a) Effect of the water plug composition on peak efficiency, (b) Effect of the water plug composition on the SEF_{height}, percentage of acetonitrile: 0, 10, 20, 30, and 40 %, water plug injection: 50 mbar 40 s. (c) Effect of the hydrodynamic injection time of water plug in the SEF_{height}, water plug composition: 30 % of acetonitrile and 70 % Milli- Q water (pH 9), hydrodynamic injection time: 0, 40, 50, 60, and 100 s at 50 mbar. Conditions: separation buffer, 50 mM SDS in 20 mM sodium dihydrogenphosphate buffer (pH 2.5) containing 30 % acetonitrile; sample concentration, 100 ng mL^{-1} in Milli-Q water (pH 9); hydrodynamic injection of 100 mM HCB for 15 s; electrokinetic injection of sample for 50 s at -20 kV; separation voltage, -27 kV.

water plug to study its effect over the enhancement of the stacking efficiency. The effect of increasing the percentage of acetonitile from 0 to 40% could be seen in Figure 3. A remarkably increase in the enhancement factor (SEF_{height}) and peak efficiency (N) was observed when the percentage of acetonitrile was increased until 30% (Figure 3a, b), but no further enhancement was achieved for higher acetonitrile percentages. As no significant differences were observed and taking into account that a higher content of acetonitrile is liable to produce air bubbles into the capillary due to the large Joule heating generated, a water solution with 30% of acetonitrile was used as the water plug in subsequent experiments. For the purpose of simplicity, subsequent mention of ‘‘water plug’’ below refers to this mixed acetonitrile/water plug.

Next, the hydrodynamic injection time or the length of the water plug was studied. The results obtained for the

enhancement factor (SEF_{height}) are shown in Figure 3c. These results indicated that by increasing the length of water plug, from 0 to 60 s, the SEF_{height} increased, whereas for higher injection times the SEF_{height} was reduced slightly.

The improvement in sensitivity was generated by the increase of the electroosmotic mobility with a relatively longer water plug. With an injection time higher than 60 s, the laminar flow originated from the mismatch of the water plug zone and the HCB solution became stronger. As a consequence, stacking efficiency decreased, resulting in a reduction in sensitivity [29]. Consequently, a water plug of 60 s, which provided the highest preconcentration effect and fairly reproducible results, was used as the optimal water plug length in the on-column preconcentration method.

3.2.5. Optimization of the Sample Injection Time and Voltage

The electrokinetic sample injection time at two different voltages, -10 and -20 kV, was also investigated as the last step of the optimization process. The SEF_{height} of the analytes increased with the electrokinetic injection time, in particular there was an improvement until 80 s at -10 kV and until 50 s at -20 kV. However, with a further increase in the injection time, the stacking efficiency began to decrease even the length of the HCB plug increased accordingly. Since analyte ions get through the boundary between the water plug and the HCB, if longer injection times are applied the sample zones become dispersed thus resulting in an incomplete stacking process [30]. These studies showed that in this report the effective injection time and voltage were 50 s and -20 kV, respectively.

To sum up, the optimum conditions were as follows: BGE composed of 20 mM NaH_2PO_4, 50 mM SDS and 30 % v/v ACN (pH 2.5); sample matrix composed of Milli-Q water at pH 9; the sample was injected using -20 kV for 50 s, after introduction of a water plug for 60 s at 50 mbar and a HCB composed by 100 mM of NaH_2PO_4, for 15 s at 50 mbar. Separation was carried out at a constant voltage of-27 kV at 25 °C.

Comparison of the electropherograms obtained from the ASEI-sweeping-MEKC and the normal-MEKC are shown in Figure 4. Under the above optimized experimental conditions, around 169 and 260-fold improvements in peak height and peak area were achieved.

3.3. Validation of the analytical method

Table 1 lists the linearity; calibration curve equations; LODs; relative standard deviations (RSDs) of the migration

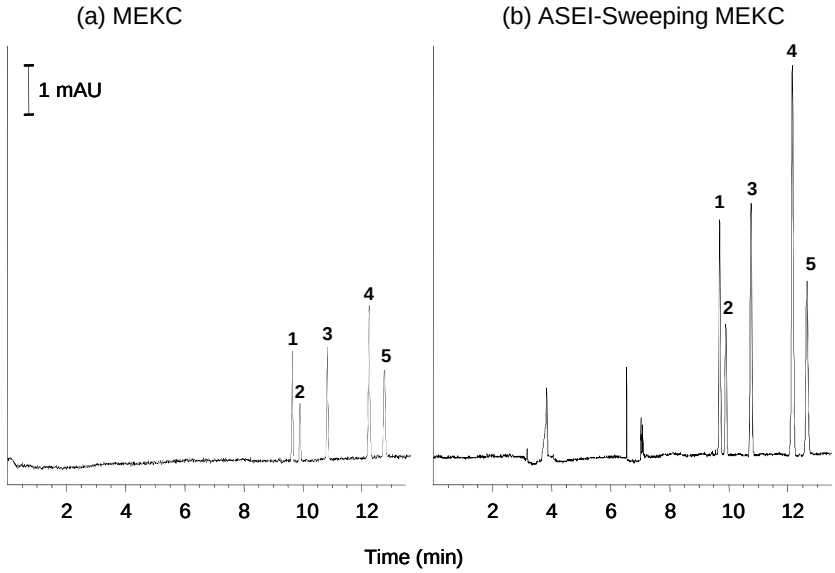

Figure 4. Electropherogram obtained from direct injection of (a) Standard sample at a concentration of 5 ng mL^{-1}: injected hydrodynamically at 50 mbar for 5 s. (b) Standard sample at a concentration of 100 ng mL^{-1} injected electrokinetically at -20 kV for 50 s. Conditions: separation buffer, 50 mM SDS in 20 mM sodium dihydrogenphosphate buffer (pH 2.5) containing 30 % acetonitrile; hydrodynamic injection of 100 mM HCB at 50 mbar for 15 s; hydrodynamic injection of water plug [Milli-Q water:ACN (70:30 v/v)] at 50 mbar for 60 s; separation voltage, -27 kV. For peak designation see Figure 2.

times, peak height and peak area; and sensitivity enhancement factor obtainned when standard samples were analyzed. The linear range in the calibration curve was 5–500 ng mL^{-1} for diclofenac and fenoprofen, 3–500 ng mL^{-1} for naproxen and 10–500 ng mL^{-1} for ibuprofen and ketoprofen, with each coefficient of determination (R^2) being > 0.999. The limits of detection (LODs, S/N = 3) for the five analytes ranged from 1.2 to 3.4 ng mL^{-1}. The day-to-day reproducibility, expressed by RSDs, was calculated by performing three replicate determinations of the same standard solution at a concentration of 100 ng mL^{-1}, in four different days (n = 4). The (RSDs) obtained by ASEI-sweeping-MEKC were lower than 0.8% for migration time, 5.6 % for peak heigh and 5.6 % for corrected peak area. The enrichment factor for each compound was within the range of 163–260 for SEF_{area} and 147–169 for SEF_{height}. These results clearly indicate that the ASEI-sweeping-MEKC method for analyzing NSAIDs provides adequate linearity, enhanced sensitivity and acceptable repeatability.

It has been the first time that ASEI-sweeping-MEKC has been applied to preconcentrate NSAIDs. The LODs ob-

tained were similar or lower to the ones obtained when different sample stacking on column preconcentration techniques (SRMM, SRMM-ASEI and FESI-RMM) combined with MEKC were used. Also it is important to remark that in the present study the analysis time was shorter (approximate 15 min) than those reported in previous studies in which they used MEKC and a stacking strategy [7].

3.4. Application

To demonstrate how the ASEI-sweeping-MEKC method could be applied for routine analysis of real samples, the method developed was validated by the analysis of river water samples. First, we carried out direct injection of river water spiked with 100 ng mL^{-1} of each of the drugs, however, the resulting separation remained poor and the sweeping effect was almost negligible, since the sample matrix composition has an important effect over this process. For that reason a dilution of the sample was necessary before the injection. To find the most suitable dilution factor, we performed different experiments. River water samples were diluted 1:2, 1:5, and 1:10 with Milli-Q water and the pH was adjusted to pH 9, and spiked with 100 ng mL^{-1} of each of the drugs. The dilution 1:10 of the river water was found to be the most suitable, giving a good separation and the best sweeping effect. Figure 5a shows the electropherogram obtained from the injection of the blank of river water diluted 1:10 to verify the absence of peaks at the same migration time of the NSAIDs, and Figure 5b shows the electropherogram obtained from the direct injection of river water diluted 1:10 and spiked with 25 ng mL^{-1} of NSAIDs.

Table 1 shows the linearity; calibration curve equations; LODs; and RSDs of the migration times, peak height and correct peak area when river water samples diluted 1:10 with Milli-Q water at pH 9, spiked with the NSAIDs at different concentrations were analyzed under the optimal ASEI-sweeping-MEKC conditions. As can be observed, the linear range in the calibration curve was 50–2,500 ng mL^{-1} for naproxen, 70–2,500 ng mL^{-1} for diclofenac and fenoprofen, and 100–2,500 ng mL^{-1} for ketoprofen and ibuprofen, with each coefficient of determination (R^2) being > 0.998. The limits of detection (LODs, S/N = 3) for the five analytes ranged from 29 to 58 ng mL^{-1}. The day-today reproducibility, expressed by RSDs, was calculated by performing three replicate determinations of the same standard solution, concentration 1,000 ng mL^{-1}, in four different days (n = 4). The RSDs obtained by ASEI-sweeping-MEKC were lower than 0.6 % for migration time, lower than 5.1 % for

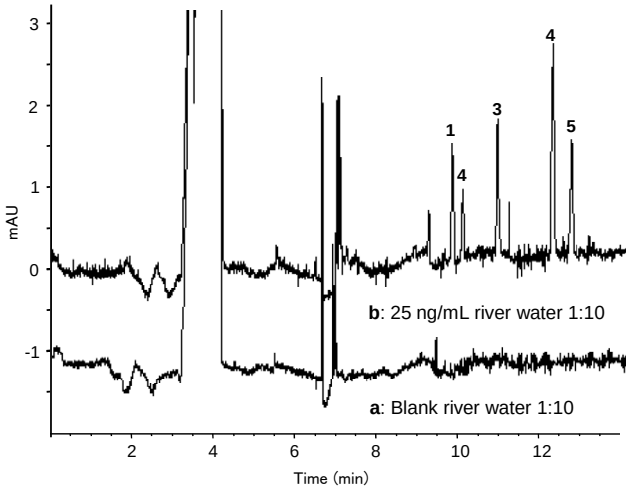

Figure 5. Electropherograms obtained from the direct injection of (a) Blank river water diluted 1:10, (b) 25 ng mL^{-1} spiked river water diluted 1:10 with Milli-Q water and then adjusted to pH 9. For other experimental conditions, see Figure 4.

peak height and lower than 4.3 % for corrected peak area.

The results obtained for the group of NSAIDs in river water samples by ASEI-sweeping technique with a previous dilution of the sample were compared to those obtained by off-line SPE followed by different preconcentration approaches based on sample stacking techniques, in-line SPE-CE or by CZE-MS. NSAIDs can be detected at a concentration range from 0.05 to 1.6 ng mL^{-1} by MEKC in tap water [7], from 0.1 to 0.250 ng mL^{-1} by MEEKC in mineral water [8], 0.1 ng mL^{-1} by CZE-MS in surface water, and 0.01 ng mL^{-1} by in-line-SPE-CE in tap water [31]. In general, the preconcentration factor for the off-line SPE is about 100-fold in all the mentioned studies. Taking into account the preconcentration factor of the off-line SPE process, the LODs achieved by sweeping with direct injection of the river water sample, ranging from 29 to 58 ng mL^{-1}, are comparable to the ones that apply SPE to analyze water samples.

4. CONCLUSIONS

We have demonstrated that a simple, rapid, and efficient ASEI-sweeping-MEKC technique may be used for on-line sample preconcentration and determination of five NSAIDs. Under the optimum conditions, the enrich-ment factors for these five compounds when using ASEI-sweeping-MEKC fell within the range 162–260 for SEF_{area} and 147–169 for SEF_{height}.

Table 1. Linearity, calibration curves, LODs, reproducibility (%RSD) of the migration time, peak height and peak area, and sensitivity enhancement factors (SEF) for standard samples and for river water samples.

Compounds	Linearity (ng/mL)	Calibration graphs			Reproducibility (%RSD)(n=4)			SEF [a,b]	
		Calibration curve	R^2	LOD (ng/mL)	Migration time	Peak height	Peak area	SEF_{area}	SEF_{height}
Standard samples									
Diclofenac sodium	5-500	y= 0.29x + 0.84	0.999	2.2	0.7	3.6	5.3	239	144
Ibuprofen	10-500	y= 0.17x + 0.48	0.999	3.4	0.8	5.6	5.0	236	169
Fenoprofen	5-500	y= 0.35x + 0.14	0.999	1.8	0.8	2.4	4.8	260	147
Naproxen	3-500	y= 0.64x + 0.30	0.999	1.2	0.8	2.2	5.6	163	163
Ketoprofen	10-500	y= 0.31x + 2.34	0.999	2.6	0.8	2.5	5.5	237	148
River water samples									
Diclofenac sodium	70-2,500	y= 0.17x + 1.13	0.999	39	0.5	3.0	2.8	-	-
Ibuprofen	100-2,500	y= 0.1x + 1.21	0.998	58	0.5	2.1	0.5	-	-
Fenoprofen	70-2,500	y= 0.22x + 0.60	0.999	41	0.6	5.1	4.3	-	-
Naproxen	50-2,500	y= 0.33x + 3.34	0.999	29	0.4	1.9	1.0	-	-
Ketoprofen	100-2,500	y= 0.20x + 1.6	0.999	38	0.4	1.2	3.5	-	-

[a] Sensitivity enhancement factor for standard samples: SEF_{area} = dilution factor × ($area_{ASEI\text{-}sweeping}/area_{normal\ hydrodynamic\ injection}$);
SEF_{Height} = dilution factor × ($height_{ASEI\text{-}sweeping}/height_{normal\ hydrodynamic\ injection}$).

[b] Sensitivity enhancement factors for river water samples were not evaluated.

It has been the first time that an ASEI-sweeping-MEKC method has been developed to determine a group of NSAIDs in river water. The proposed method allows the separation and preconcentration of five NSAIDs in river water in < 15 min with LODs ranging between 29 and 58 ng mL^{-1}. The results obtained for this group of NSAIDs in river water samples by applying an ASEI-sweeping technique with a direct injection of the sample are comparable to those reported in the bibliography, in which other on-column preconcentration CE techniques have been used [7,8,31]. Furthermore, it is remarkable than they use an off-line sample pretreatment and preconcentration.

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3.1.3. Discussion of results

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The results obtained in the two studies developed in this section show the potential of CE when combined with preconcentration techniques, such as sweeping and ASEI-sweeping, for the analysis of NSAIDs in water samples.

In the case of sweeping preconcentration, the optimal conditions for the micellar electrophoretic separation were taken from a previous study carried out by our group [1], which focused on the determination of a group of NSAIDs in water samples using MEKC under pH-suppressed EOF. The study was developed with a capillary with an I.D. of 75 µm. The BGE was composed of 25 mM sodium dihydrogenphosphate and 75 mM SDS with 40 % of ACN at pH 2.5, which is a suitable BGE for application to the sweeping methodology. The parameters that were optimized for sweeping were the composition of the sample matrix and the sample injection time. As reported previously by Palmer *et al.* [2] and Quirino *et al.* [3], the conductivity of the sample matrix is a key parameter to be optimized, as differences in the conductivity values between the sample and the BGE involve a combination of sweeping and stacking mechanisms. The conductivity ratio between the sample and the BGE (R=sample conductivity/BGE conductivity) was then studied, obtaining the best results in terms of sensitivity enhancement peak height and peak efficiency when the conductivity of the sample matches the conductivity of the BGE (R=1). Injection time affects the amount of sample introduced in the CE and hence has a direct influence on sample preconcentration. The sample solution was injected at different times and for injections longer than 350 s the current values were relatively higher. An injection time of 350 s gave the best focusing effect with proper peak separation and efficiency.

To improve the preconcentration factors achieved by sweeping, in the second study, sweeping was combined with field-enhanced sample injection (ASEI-sweeping). This preconcentration technique is based on a selective electrokinetic injection followed by the addition of surfactant to carry out the sweeping process. As the sweeping study was carried out under relatively high current values, in order to develop the ASEI-sweeping methodology, a capillary with a shorter I.D. of 50 µm was chosen in order to avoid problems with high current values. The optimized BGE composition for the capillary of 50 µm was 20 mM sodium dihydrogenphosphate and 50 mM SDS with 30 % of ACN at pH 2.5. The parameters affecting both focusing mechanisms, namely stacking and sweeping, were investigated and optimized. These include the SDS concentration, the composition of the sample matrix, the concentration and the injection length of the HCB, the composition and injection length of the water plug, and finally the sample injection time and voltage. It should be pointed out that the water plug and the HCB plug improve the ASEI step by creating a long sample zone with a higher concentration

than in the original solution, but they do not contribute to the focusing effect of the analyte via sweeping. The water plug during the ASEI step helps to maintain a high electric field at the tip of the capillary, which allows the anionic analytes to enter the capillary at high velocity. The presence of the HCB improves the total stacking effect of the analytes as it acts as a wall due to the high conductivity differences between the water plug and the HCB. The sample matrix conductivity which affects the injection of the sample and also the sweeping focusing was first optimized. For the electrokinetic injection of the analytes by ASEI, the analytes should be in their ionic form and in a matrix with low conductivity. This was achieved when the sample was prepared with Milli-Q water adjusted to pH 9.0. The results of the optimization of the SDS concentration were achieved through a compromise between preconcentration of the analytes and resolution of the peaks, as the SDS concentration affects the electrophoretic separation and also the preconcentration of the compounds. The optimal HCB and water plug effect was achieved when 100 mM of sodium dihydrogenphosphate (HCB) was injected for 15 s, followed by the injection of a plug of water with 30 % of ACN (water plug) for 60 s. Finally, the amount of analytes introduced into the CE was evaluated. This was determined by the anodic voltage applied and the length of time it was applied. The highest sensitivity enhancement factors were obtained when the sample was injected at -20 kV for 50 s.

In order to compare the sensitivity enrichment factors achieved by both methods, sweeping was also evaluated with a capillary with an I.D. of 50 µm. Using the BGE optimized for ASEI-sweeping and a sample matrix with the same conductivity as the BGE, the hydrodynamic injection time of the sample was optimized and the best focusing effect with proper peak separation and efficiency was achieved by a sample injection time of 300 s.

Table 3.1 shows the range of sensitivity enrichment factors (SEF) in terms of peak area (SEF_{area}) and peak height (SEF_{height}) and the limits of detection (LODs, S/N = 3) obtained for sweeping and for ASEI-sweeping.

As can be observed in the table, the preconcentration improvement by ASEI-sweeping compared to sweeping is clearly demonstrated through the SEF obtained by both techniques using the same capillary system, which show that the preconcentration factors obtained by ASEI-sweeping (SEF_{height} = 144- to 169-fold, SEF_{area} = 163- to 260-fold) are double the preconcentration factors obtained by sweeping (SEF_{height} = 51- to 79-fold, SEF_{area} = 82- to 120-fold).

Table 3.1 SEFs and LODs for the studied preconcentration strategies.

	Sweeping	**Sweeping**	**ASEI-sweeping**
Capillary	75 µm I.D., 60 cm length	50 µm I.D., 60 cm length	50 µm I.D., 60 cm length
Analysis time	20 min	18 min	15 min
Injection	50 mbar 350 s (2.3 µL)	50 mbar 300 s (0.4 µL)	-20 kV 50 s
SEF_{height}	113-163	51-120	144-169
SEF_{area}	298-401	82-260	163-260
LODs (ng mL^{-1})[a]	1.2-2.5	--	1.2-3.4
LODs (ng mL^{-1})[b]	6.5-14.6	--	29-58

[a] Standard samples
[b] River water samples

Both methodologies were applied to the determination of NSAIDs in river water samples. In both cases, the samples were diluted and then injected directly into the system without further sample pretreatment. In the sweeping method, the sample was diluted 1:5, and in the ASEI-sweeping method, the sample was diluted 1:10. A higher dilution was performed for the ASEI-sweeping as a cleaner sample matrix was needed in order to carry out the electrokinetic injection of the sample. In the conditions studied in both preconcentration strategies, the LODs achieved for river water samples were in the range of 6.5 ng mL^{-1} to 14.6 ng mL^{-1} for sweeping, and in the range of 29 ng mL^{-1} to 58 ng mL^{-1} for ASEI-sweeping. These differences in the LODs are mainly due to the different dilution factors of the samples, as in the case of ASEI-sweeping, the water sample was diluted by double the amount as in the case of sweeping, as well as to the different I.D. of the capillaries used in each methodology.

The LODs obtained by both methodologies are comparable to those reported in the literature when other preconcentration techniques, such as sample stacking techniques, EKS and in-line SPE-CE, are applied for the determination of NSAIDs in standard samples [1, 4-7]. In contrast, when sweeping and ASEI-sweeping are applied to the analysis of environmental waters, the LODs obtained are at least one or two orders of magnitude higher than the previously mentioned studies. However, it should be emphasized that in some of the above mentioned works, a preconcentration step based on the use of off-line SPE was applied which led to a higher degree of preconcentration.

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3.2. Determination of pharmaceutical and personal care products by in-line solid-phase extraction-capillary electrophoresis

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Solid-phase extraction is the most widely used technique for the preparation of environmental liquid samples because of the wide range of commercially available sorbents. SPE can be used to clean the sample matrix, as well as for preconcentrating and storing the sample. Generally, SPE is used in an off-line mode. However, over recent years, it has also been performed in-line. In other words, SPE is directly integrated into the analytical method, in combination with either chromatographic or electrophoretic techniques [1-7].

The in-line coupling of SPE with CE has a great number of advantages in comparison with other combinations of both techniques, such as not involving the modification of the CE system. The studies presented in this section focus on the development of two in-line SPE-CE strategies for the preconcentration and determination of pharmaceuticals and personal care products in river water samples. From the different devices developed in previous studies reported in the literature involving in-line SPE-CE, the packed bed design was used in our study. In particular, an analyte concentrator was constructed using SPE sorbent positioned between two capillaries without the need of frits, as the sorbent particles size was larger than the I.D. of the separation capillary. Our aim was to develop an in-line system without frits in order to avoid bubble formation leading to current disruption and irreproducibility of the migration times.

As mentioned in the introduction, most of the PPCPs that reach wastewater treatment plants are not completely removed and so they may be present in the aquatic environment [8,9]. Consequently, there is a growing need to develop reliable analytical methods, which enable a rapid, sensitive and selective determination of these PPCPs in environmental water samples.

The compounds preconcentrated and determined in the first study were a group of pharmaceutical compounds, including certain NSAIDs (piroxicam, diclofenac sodium, naproxen) as well as some lipid regulators (bezafibrate and clofibric acid). The importance of these compounds has already been mentioned in the previous section. In the second study, four UV-filters (benzophenone-3 (BP-3), 2,2-dihydroxy-4-methoxybenzophenone (DHMB), 2,4-dihydroxybenzophenone (DHB), and 2-phenylbenzimidazole sulphonic acid (PMDSA)) were preconcentrated and separated, all of which belong to the group of PCPs group. UV-filters are chemical compounds that can filter UV-A and/or UV-B radiation from sunlight in order to shield human skin from their negative effects. UV-filter compounds are integrated into many cosmetic formulations (e.g. sunscreen creams, lotions, shampoos, lipsticks, hairsprays, hair dyes, etc.) and they reach surface waters (rivers, lakes, coastal sea water) via release from the skin during swimming and bathing or through wastewater. Most UV filters are highly lipophilic and

hardly degradable in sewage treatment plants and are therefore bound to accumulate in the environment [10].

In the first study, the suitability of a method based on in-line SPE-CE with UV-Vis detection was evaluated for the preconcentration and separation of the above mentioned pharmaceutical compounds. The sorbent used to construct the in-line SPE-CE was the reversed-phase sorbent Oasis HLB, as it is an universal sorbent for acidic, basic, and neutral compounds and, as reported in the literature, this sorbent has been widely used for the off-line SPE of these kind of compounds with good recoveries [11]. Different parameters affecting the in-line SPE-CE, such as sample pH, volume of the elution plug, and sample loading time were studied in order to obtain the maximum preconcentration factors. The developed methodology was applied for the analysis of river water samples fortified with the pharmaceutical compounds.

In the second study, in-line SPE-CE coupled to a more sensitive and selective detector than UV-Vis detector, namely the MS detector, was investigated for the preconcentration and separation of four UV-filters. In this case, three different sorbents were evaluated and compared: a reversed-phase sorbent (Oasis HLB), a mixed-mode anion-exchange and reversed-phase sorbent (Oasis MAX), and a mixed-mode cation-exchange and reversed-phase sorbent (Oasis MCX). These sorbents were selected as they have been used in an off-line SPE mode followed by LC in order to extract and preconcentrate PCP compounds, including UV-filters [11]. As in the previous study, the main parameters affecting the preconcentration performance, such as sample pH, volume and composition of the elution plug, and sample injection time were studied. The applicability of the developed methodology was demonstrated by the analysis of these compounds in river water samples with a previous off-line SPE pretreatment of the sample.

The results of the first study have been published in *Electrophoresis* 32 (2011) 2114-2122. The results of the second study have been submitted for publication to the same journal. The second article has been developed in collaboration with Professor Christian Neusüß of the Chemistry Department of Aalen University (Germany).

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3.2.1. An in-line SPE strategy to enhance sensitivity in CE for the determination of pharmaceutical compounds in river water samples

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AN IN-LINE SPE STRATEGY TO ENHANCE SENSITIVITY IN CE FOR THE DETERMINATION OF PHARMACEUTICAL COMPOUNDS IN RIVER WATER SAMPLES

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Abstract

In this study, the suitability of solid-phase extraction (SPE) coupled in-line to CE with UV–Vis detection was evaluated for the preconcentration and separation of diluted solutions of five pharmaceuticals compounds: bezafibrate, piroxicam, diclofenac sodium, naproxen and clofibric acid. A SPE analyte concentrator (AC) containing Oasis HLB sorbent was constructed without frits and placed near the inlet end of the separation capillary. Different parameters such as sample pH, volume of the elution plug, and sample loading time were studied in order to obtain the maximum preconcentration factors. The detection limits (LODs) reached for standard samples were in the range 0.06-0.5 ng/mL with good reproducibility, and the developed strategy provides sensitivity enhancement factors around 14000-fold in peak area and 5900-fold in peak height compared with normal hydrodynamic injection. Finally, river water samples fortified with the pharmaceutical compounds were analyzed by the developed in-line SPE-CE-UV method in order to show the potential of the methodology for the analysis of environmental aquatic samples. For these samples, high values of relative recoveries, between 73-107 % and 79-103 % for two concentration levels, 5 and 25 ng/mL, respectively, were obtained and LODs ranged between 0.19-1.0 ng/mL.

Keywords: In-line solid phase extraction; capillary electrophoresis; pharmaceutical compounds; river water samples.

1. INTRODUCTION

Large amounts of pharmaceutical compounds (e.g. antiinflamatories, lipid regulators, antiepileptic drugs, antibiotics, etc) are used worldwide and they can be present in the aquatic environment. In fact, they can reach wastewater treatment plants from sewage water where it has been shown

that they are not completely removed [1], and in this way they can be present in surface waters. The concentrations of individual compounds in surface waters are typically in the range of several tens to hundreds of ng/L, although concentrations at the μg/L level are also reported for some compounds and specific sites [2-7].

Specific analytical methods are required to determine these type of compounds in environmental waters and, in this sense, there are different approaches, such as the based on high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE) [1-4].

The application of capillary electrophoresis (CE) for the analysis at low concentration levels has some limitations due to the poor sensitivity of this technique. In order to expand the use of CE for trace analysis, several strategies have been reported [4,5]. Among them, one of the most widely used is been based on the use of a chromatographic preconcentration technique such as a solid-phase extraction (SPE) procedure. SPE can be combined with CE in off-line, at-line, in-line or on-line modes [6-8].

In in-line SPE-CE, the SPE sorbent or analyte concentrator (AC) is fully integrated into the CE configuration and the separation voltage is applied across the SPE material. This in-line mode has some advantages over the other configurations. Mainly, there is no need for modification of the commercial CE instrumentation like those required for at-line or on-line systems, avoids tedious sample-handling steps and makes the analysis faster. Another advantage is that the complete eluate from the SPE is analysed by CE. Finally, the small quantity of sorbent material needed together with the lower consumption of sample and organic solvent makes in-line SPE cheap and environmentally friendly [6]. In spite of the great benefits of in-line SPE, this is not a widely used technique maybe because ACs are not commercially available so they have to be constructed by the user. Moreover, the separation efficiency greatly depends on the volume and the nature of the elution solvent used. Another disadvantage is that the sample matrix components also pass through the separation capillary and may interfere with the CE analysis, or they can be adsorbed onto the capillary wall, which can result in poor separation.

In general, in-line SPE-CE can be performed by using different strategies: one is the use of an open tubular (OT) capillary in which the SPE sorbent is coated in the capillary walls [9-11]; another is the use of a packed bed [12-21] or monolithic material [22-25], in which a SPE microcartidge or analyte concentrator (AC) containing

the packing material sorbent, is kept near the inlet side of the CE capillary; and the last one is the use a thin impregnated membrane or SPE material positioned between two capillaries [26].

Among all these alternatives, the packed-bed design has been the most commonly used. In this configuration, generally, the SPE material is retained by means of two frits to prevent the sorbent particles from entering and blocking the CE capillary. Some of the different compounds that have been determined with this approach are peptides in human plasma, using a microcartridge with C18 sorbent [14,17], ochrotoxin, naproxen, ceftiofur and chlorophenols in river water samples, also using C18 as SPE sorbent [13,15,16,21] and 3-nitrotyrosine in rat urine by using Oasis MCX sorbent [20]. Although frits are normally needed to prevent sorbent looses, and they are relatively easy and fast to built, their presence can produce an increased backpressure, a disturbance of the EOF and also they can induce bubble formation during the CE separation, leading to band broadening, current disruptions, and irreproducibility of the migration time. To overcome these problems, some authors have investigated an alternative in the packed bed configuration in which they do not use frits. For example, Lara *et al.* [12,18,19] used sorbent particles with higher sizes than the internal diameter of the separation capillary. In one of their papers, the authors developed and validated an in-line SPE-CE-MS method for the determination of eight quinolone antibiotics in chicken muscle [19], using a capillary of 50 µm of I.D. and a mixed-mode Oasis MCX sorbent with an average particles size of 60 µm, and in this case the LODs obtained ranged between 17 and 59 ng/L for standard solutions. The same authors developed another in-line SPE-CE-UV method for the determination of triazine herbicides in human urine [18], using a capillary of 50 µm of I.D. and as a SPE sorbent they used a commercial moleculary imprinted polymer (MIPs) with an average particles size of 55 µm. In this study the obtained LODs were between 200 and 600 ng/mL. Finally, the same authours developed another in-line SPE-CE-UV strategy for the determination of sulfonamides in water samples [12], using a capillary of 50 µm of I.D. and an hydrophilic-lipophilic-balance Oasis HLB sorbent with an average particles size of 60 µm, with LODs between 0.23 and 0.48 ng/mL .

The main aim of this work has been to develop a frit-free in-line SPE device for capillary electrophoresis for the preconcentation and separation of a group of acidic pharmaceutical compounds (bezafibrate, piroxicam, diclofenac sodium, naproxen and clofibric acid). Different parameters such as sample

pH, the elution plug volume, the sample loading time, and the washing step, which affect the preconcentration enhancement factors and the separation, were optimized. Finally, the developed in-line SPE-CE methodology has been tested for the analysis of river water samples fortified with the pharmaceutical compounds.

2. MATERIAL AND METHODS

2.1. Standards and reagents

Standards of the five pharmaceutical compounds (bezafibrate, piroxicam, diclofenac sodium, naproxen, and clofibric acid) and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide (98 %) was from Panreac (Barcelona, Spain), acetic acid and ammonium hydroxide (25 %) were from Scharlau (Barcelona, Spain), and methanol was from SDS (Peypin, France). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Oasis HLB sorbent, with an average particles size of 60 µm, was supplied by Waters (Milford, MA, USA).

A stock standard solution of 1000 mg/L for each pharmaceutical compound was prepared in methanol. A mixed standard solution of the five compounds was prepared weekly at a concentration of 100 mg/L in methanol. The working standard solutions were prepared daily by diluting appropriate volumes of the standard solutions with Milli-Q water. All solutions were stored in a dark-glass flask at 4 °C. The background electrolyte (BGE) solution was prepared daily, and all buffers and working solutions were sonicated and filtered through a 0.22 µm nylon syringe filter (Tracer, Teknokroma, Barcelona, Spain) before used.

2.2. Instrumentation

CE analyses were performed on a Hewlett-Packard 3D CE instrument (Agilent Technologies, Waldbroon, Germany) system equipped with a UV diode-array detector (DAD) operating at 214 nm. Bare fused-silica capillaries with 50 and 150 µm I.D. were purchased from Agilent Technologies (Waldbroon, Germany). The capillary temperature was set at 25 °C for all the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used.

2.3. CE procedure

Capillaries of 112.5 cm total length (104 cm effective length) and 50 µm I.D. were used for CE separation. The BGE consisted of an aqueous solution of 20 mM of ammonium acetate adjusted at pH 9 with 25 % ammonium

hydroxide. The separation voltage was 28 kV. Before the first use, the capillaries were conditioned by flushing with 1 M NaOH for 15 min, then with water for 20 min, and finally with the BGE solution for 30 min. At the beginning of each day, the capillaries were conditioned for 5 min with 0.1 M NaOH, for 5 min with water and finally with running buffer for 10 min. After each injection the capillary was conditioned with 0.1 M NaOH for 1 min, with water for 1 min and finally with running buffer for 3 min. A pressure of 930 mbar was applied in all cases. Hydrodynamic injections were carried out by applying a pressure of 50 mbar for 5 s.

2.4. Construction of the analyte concentrators

The analyte concentrator which contained the SPE sorbent was home-made. First, a 500 mg Oasis HLB cartridge (60 µm of particle size) was opened, their frits were removed, and finally the sorbent was transferred into a pre-cleaned glass vial. On the other hand, a 112.5 cm length bare fused-silica capillary (50 µm I.D. × 360 µm O.D.) was conditioned with 1 M sodium hydroxide for 20 min and then with Milli-Q water for 15 min at 930 mbar. Then, it was cut into two pieces of 7.5 (inlet capillary) and 105 cm (separation capillary), respectively, in order to insert the AC between them. Following, the AC was prepared cutting 2 mm of bare fused-silica capillary of 150 µm I.D. and 360 µm O.D. A proper cut on both sides of the capillary is essential to obtain an optimum performance of the concentrator. The AC was introduced 1 mm into a 0.5 cm piece of PTFE tubing (Grupo Taper S.A., Madrid, Spain) with an I.D. of 0.250 mm. PTFE material can expand to fit the outer diameter of the bare fused-silica capillary. Then, the piece of 7.5 cm was introduced at the other end of the PTFE tubing until connect with the AC (inlet), and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into the vial that contained the Oasis HLB sorbent, and this was loaded into the AC. Then, the capillary of 7.5 cm and the AC were moved until the preconcentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 105 cm was introduced into the other part of the PTFE tubing until to join the other side of the AC (outlet). The entire process of fabricating the concentrator was monitored under a microscope. Figure 1A shows the described design.

Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow by filling the capillary with water and applying a pressure of 930 mbar to a vial containing MeOH.

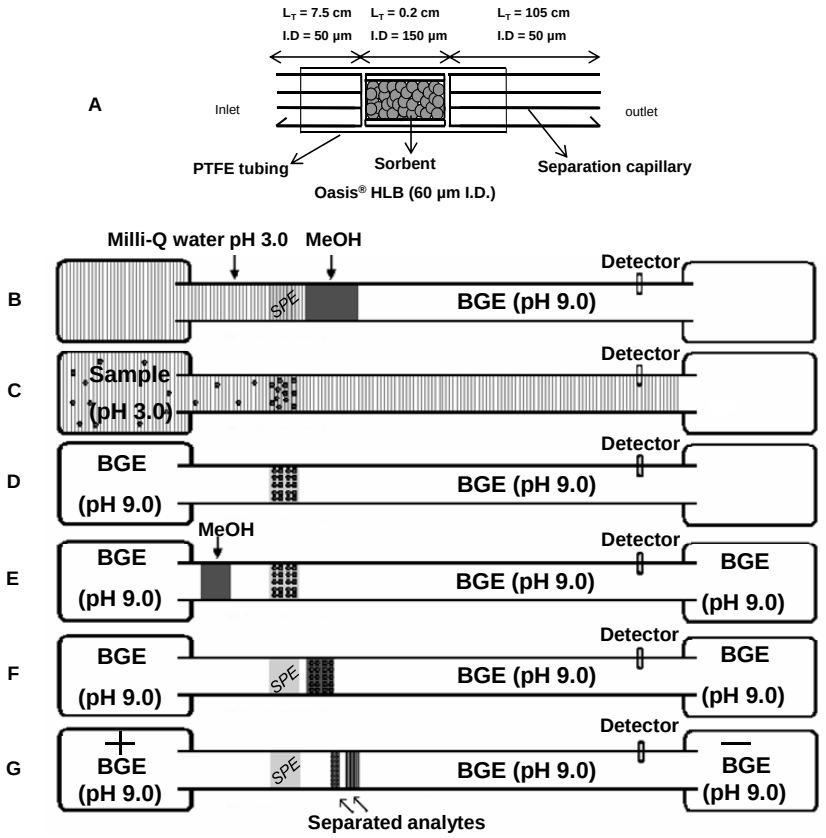

Figure 1. (A) Schematic diagram of the in-line SPE-CE device. From (B) to (G) the different steps followed to preconcentrate and separate the analytes are shown: (B) the SPE sorbent was first wetted and conditioned with methanol for 1 min and Milli-Q water for 2 min at 930 mbar, respectively; (C) then the sample was loaded at 930 mbar for 60 min; (D) a rinse with BGE for 3 min at 930 mbar was performed and subsequently, (E) the elution of the analytes with methanol, injected for 50 s at 50 mbar took place. Then (F) the introduction of BGE at 50 mbar for 230 s to push the elution plug through the concentrator was carried out. Finally, (G) a voltage of 28 kV was applied between two vials filled with BGE to perform the electrophoretic separation.

The time needed for MeOH to reach the detector was measured by monitoring the signal at 214 nm. This time was about 107 s in a properly constructed capillary (in-line SPE-CE), which is the same that in a capillary without an in-line SPE sorbent.

2.5. In-line SPE-CE procedure

The capillary was conditioned with BGE for 15 min at the beginning of each day. The different steps of in-line SPE-CE procedure are shown in Figure 1. First, (B) the SPE sorbent was wetted

with methanol for 1 min at 930 mbar in order to remove trapped air and solvate the sorbent, then, it was conditioned by flushing acidic Milli-Q water (pH 3) (after optimization) for 2 min at 930 mbar; (C) the sample was then loaded at pH 3 at 930 mbar for 60 min (ca. 46 µL using Hagen-Poiseuille equation); (D) the next step was a sample clean up with BGE solution by applying 930 mbar for 3 min; (E) afterwards, the retained analytes were eluted by injecting a plug of methanol, using a pressure of 50 mbar for 50 s (34 nL) and (F) followed by BGE at 50 mbar for 230 s to push the elution plug through the concentrator until the beginning of the separation capillary (ca. 8 cm using Hagen-Poiseuille equation). Finally, (G) a voltage of 28 kV was applied between two vials filled with BGE to perform the electrophoretic separation. After each injection the capillary was washed with methanol for 2 min to avoid carry-over problems between consecutive injections.

2.6. Sample pretreatment

Water samples were collected from Ebro River, Catalonia (Spain). All samples were collected by using pre-cleaned amber glass bottles and then they were acidified to pH 3 with HCl, filtered through a 0.22 µm nylon syringe and stored at 4 °C until their analysis.

3. RESULTS AND DISCUSSION

3.1. CE separation

The CZE separation was performed with a BGE composed by 20 mM of ammonium acetate at a pH value of 9 to ensure the total ionization of the studied compounds [27]. The hydrodynamic injection of the standard samples was carried out by applying a pressure of 50 mbar for 5 s. Under these conditions, when the positive voltage is applied, the anionic compounds migrate to the detection window by the EOF mobility, while their own electrophoretic mobility is in the opposite direction. An efficient separation, with a good resolution, was obtained in less than 18 min for all the compounds.

3.2. Optimization of the in-line SPE process

In this study, Oasis HLB sorbent was used as the extraction sorbent. This is a hydrophilic-lipophilic-balanced polymeric SPE sorbent, useful for a broad chromatographic polarity range of acidic, basic, and neutral analytes. Different authours have previously used this sorbent in the solid-phase preconcentration of this kind of pharmaceutical compounds. For example, Pedrouzo *et al.* [28] determined some of these compounds in river and

sewage treatment plant (STP) waters by HPLC-MS, achieving recoveries between 59 and 89 % for bezafibrate, and between 33 and 61 % for clofibric acid. Sebők *et al.* [29] determined a group of NSAID, among them naproxen and diclofenac sodium, in water samples from a sewage treatment plant by GC-MS, achieving recoveries between 96 and 103 % for both compounds. Recently, Gracia-Loc *et al.* [30] have determined some of these compounds in river and sewage treatment plant waters by UPLC-MS/MS achieving recoveries between 84 and 103 % for naproxen, 86 and 99 % for bezafibrate, and 84 and 98 % for diclofenac sodium. In all of the developed methodologies for the mentioned authors, methanol was the selected elution solvent.

In this work, several parameters were investigated to achieve the optimum performance for the in-line SPE-CE method, including the sample pH, the volume of the elution plug, the sample loading time, and the washing step.

3.2.1. Sample pH

The sample pH involves a different ionization degree of the compounds, and it plays an important role in the retention of the analytes into the sorbent. Since the pKa values of this group of compounds ranged from 3.46 to 6.3, we examined the influence of having a lower, similar and higher pH value for the sample than the pKa values. To do that, the analytes were prepared in Milli-Q water at pHs values of: 1.5, 3.0, 5.5, 9.0 and 10.5. In all the cases, first the AC was wetted and conditioned with methanol for 1 min and Milli-Q water at the same pH than the sample for 2 min at 930 mbar, in order to remove trapped air and activate the SPE sorbent. The standard solutions were then loaded into the capillary at 930 mbar for 5 min. Afterwards, the AC was washed by flushing with the BGE solution for 5 min at 930 mbar. Finally, the drugs were eluted with methanol for 30 s at 50 mbar, followed by BGE at 50 mbar for 230 s to push the elution plug through the concentrator until the beginning of the separation capillary (ca. 8 cm using Hagen-Poiseuille equation). The electrophoretic separation took place at 28 kV. The results are shown in Figure 2A, where the response for the different compounds, in terms of peak area, is represented at the different pH values tested. As can be observed in this figure, the higher retention in the Oasis HLB sorbent was obtained at acidic pH values (pH 1.0 and 3.0). At this pH range, compounds are in their nonanionic form, they are retained primarly by the strength of their hydrophobic interaction with the sorbent face [28,29]. Finally we choose a pH of 3 since slightly better results in terms of peak area were obtained.

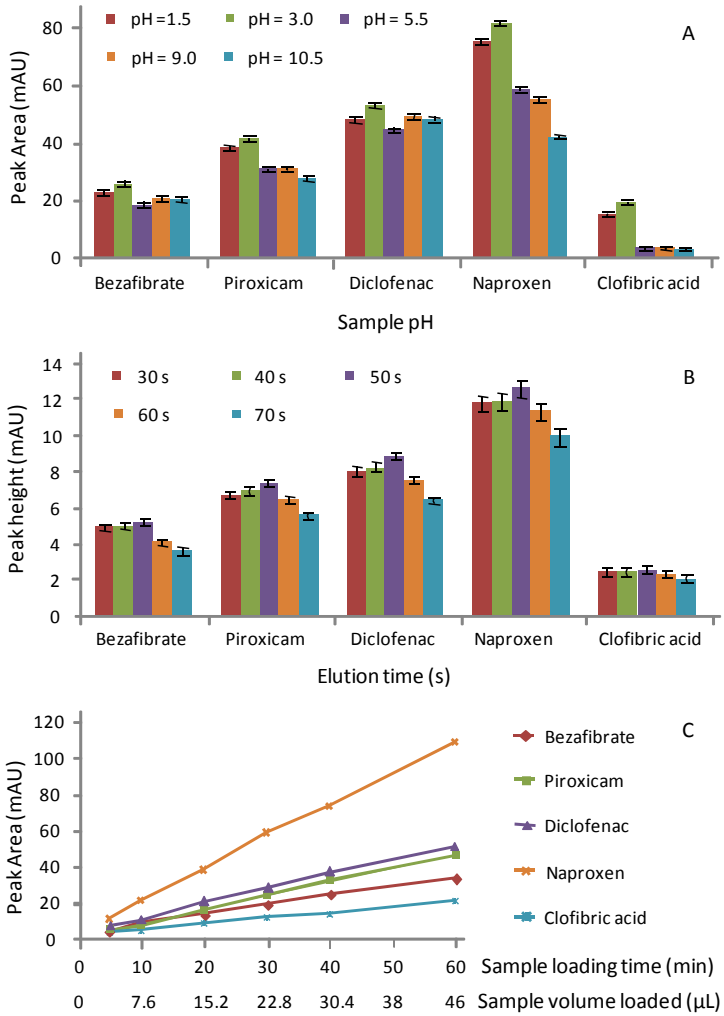

Figure 2. Effect of different parameters on the performance of the developed in-line SPE-CE method for the determination of a group of five acidic drugs. (A), Effect of the sample pH on the peak area, sample prepared at a concentration of 50 ng/mL (B) Effect of the volume of the elution solvent, which was methanol, on the peak height, sample prepared at a concentration of 50 ng/mL, and (C) study of the sample loading time, sample prepared at a concentration of 1 ng/mL. Other experimental conditions are reported in the text. In all the cases the experiments were performed by triplicate.

3.2.2. Elution step

In our case methanol was the elution solvent since, as reported in the bibliography, this is the preferred choice for the elution of polar compounds when Oasis HLB is the used sorbent, due to its high elution power. Moreover, we can highlight that this solvent was previously used for the

determination of this group of compounds by an off-line SPE with the same sorbent [28-30]. The elution volume was studied by introducing MeOH with a hydrodynamic injection from 30 to 70 s (20 to 48 nL) at 50 mbar. Figure 2B shows that the peak heights for the different compounds slightly increase with the elution volume until 50 s (34 nl). When higher plugs of methanol were used, a loss of efficiency and resolution were observed, which involved a decrease in the peak height. Taking into account this, we selected an elution volume of 34 nL (50 s).

3.2.3. Sample loading time

The introduction of a large sample volume is the most important factor to obtain lower LODs. In order to load the maximum sample volume and establish the maximum capacity of the Oasis HLB sorbent, the sample loading time at 930 mbar was investigated using a solution of 1 ng/mL of naproxen. Figure 2C shows the peak area of the eluted compounds as a function of the sample loading time or the sample volume loaded, which was increased from 5 to 60 min (i.e. 4 - 46 µl) at 930 mbar. A linear relation of the peak area versus loading time was observed until 60 min, that's mean that the breakthrough volume was not exceeded. Although we observed this trend of increasing the response with higher injection volumes of the sample, we selected as the optimum value an injection time of 60 min. We also considered for this choice that the introduction of a higher sample volume would prolong too much the analysis time.

3.2.4. Clean-up step

Once the sample was successfully loaded, a final rinse with BGE was necessary, as well as to remove unretained molecules, also to fill and conditioning the capillary before the electrophoretic separation. We tested different rinsing times, from 3 to 6 min, and we observed that the decrease of the rinse didn't affect the peak area of the eluted compounds. To minimize analysis time, the rinse with BGE was performed for 3 min. This parameter was also carefully considered for the evaluated real samples in the present study, in particular river water samples. For these real samples, the complexity of the matrix is higher, and it can involve the need of a higher rinsing time with the BGE to clean the sorbent and to condition again the capillary before the electrophoretic separation.
To sum up, the optimum conditions were as follows: BGE composed of 20 mM ammonium acetate pH 9; sample dissolved in Milli-Q water at pH 3; the AC was wetted and conditioned with

methanol for 1 min and Milli-Q water pH 3 for 2 min at 930 mbar; the sample was injected at 930 mbar for 60 min; the AC was cleaned-up by flushing with the BGE solution for 3 min at 930 mbar; the compounds were eluted with methanol for 30 s at 50 mbar, followed by BGE at 50 mbar for 230 s. The electrophoretic separation took placed at 28 kV.

An important consideration is the AC life-time. In this point we would like to emphatise that it is necessary that the concentrator is changed when an important shift on migration times occurs between different analysis. In this study, to perform all the experimental work, we used two different AC.

Figure 3A shows the electropherogram obtained when a 10 µg/mL standard solution of the pharmaceutical compounds was analysed by CE with normal hydrodynamic injection (50 mbar for 5 s). Figure 3B shows the electropherogram obtained under the optimum in-line SPE-CE conditions, when a standard solution of the pharmaceutical compounds at pH 3.0 at a concentration of 10 ng/mL was loaded at 930 mbar for 60 min. As it can be observed from the comparison of both electropherograms, a considerable enhancement of the concentration sensitivity is achieved using the developed methodology based on in-line SPE-CE. We obtained around 5900- and 14000 fold improvements in peak height and peak area, respectively. However, a slight decrease in the peak efficiency and resolution were also observed. For the CZE, an efficiency range between 410000 and 710000 and a resolution between 4 and 10 were obtained, whereas in the case of the in-line SPE-CE the efficiency range was between 94000 and 220000, and the resolution between 2 and 5. The decrease in peak efficiency and resolution can be attributed to the presence of the AC in the separation capillary.

3.3. Validation of the analytical method

Under the optimum conditions, the method was evaluated in terms of linearity, limits of detection (LODs), repeatability, reproducibility, sensitivity enhancement factors (SEFs) and recoveries (Table 1). The linear range in the calibration curve was 1-25 ng/mL for bezafibrate, 0.5-25 ng/mL for piroxicam and diclofenac sodium, 0.25-25 ng/mL for naproxen, and 2.5-25 ng/mL for clofibric acid, with each coefficient of determination (R^2) being greater than 0.996. The limits of detection (LODs, S/N = 3) for the five analytes ranged from 0.06 to 0.50 ng/mL. The day-to-day reproducibility, expressed in terms of relative standard deviation (RSDs), was calculated by

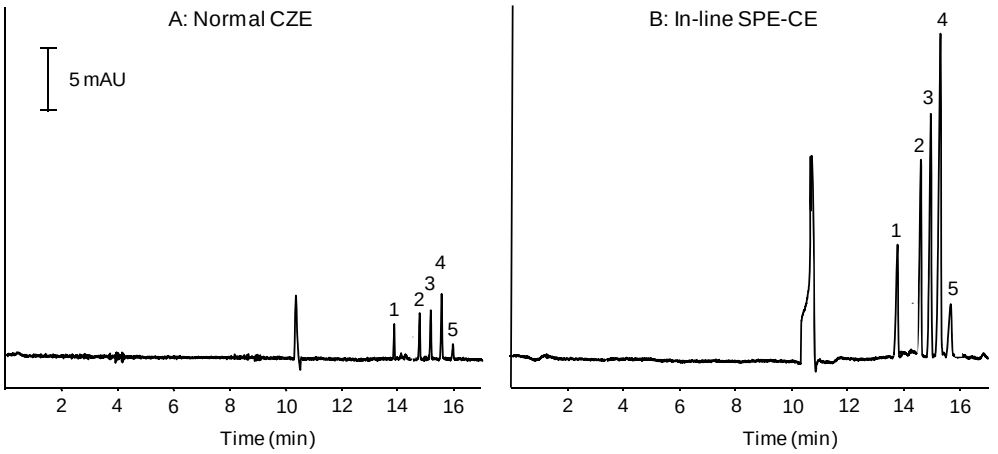

Figure 3. Electropherograms obtained after analyzing (A) a standard solution containing the acidic drugs, at a concentration of 10 µg/mL and injected hydrodynamically at 50 mbar for 5 s, by CZE, and (B) a standard solution of the acidic drugs, at a concentration of 10 ng/mL injected at 930 mbar for 60 min,by in-line-SPE-CE. Peak designation: (1) bezafibrate; (3) piroxicam; (3) diclofenac sodium; (4) naproxen; and (5) clofibric acid. Other experimental conditions are reported in the text.

performing three replicate determinations of the standard solution at a concentration of 10 ng/mL, in three different days (n=3). The RSDs obtained by in-line SPE-CE were lower than 5.4 % for migration time, 6.5 % for peak height and 5.3 % for peak area. The repeatability, expressed in terms of relative standard deviation (RSDs), was evaluated by three replicate experiments (n=3) at a concentration of 10 ng/mL. The (RSDs) obtained by in-line SPE-CE were lower than 1.8 % for migration time, 2.1 % for peak height and 2.4 % for peak area. The SEF values for each compound were within the range of 11500-14000 for SEF_{area} and 4700-5900 for SEF_{height}. Absolute recoveries were calculated as an estimation of the efficiency of an AC. To carry out this evaluation, the same moles of analytes are injected in both, an electrophoretic capillary incorporating the proposed AC and a similar capillary but without the preconcentrator. It is not expected to loss the analytes during the injection step in the normal capillary without the AC, i.e., all the molecules injected are going to be detected. However, this is not the case of real ACs; therefore, peak areas were compared in both capillaries to calculate the in-line SPE recoveries. We evaluated recoveries at two concentration levels, 5 ng/mL and 25 ng/mL, and values between 82 % and 116 % were obtained.

These results clearly indicate that the developed in-line SPE-CE method for analyzing this group of pharmaceutical

Table 1. Linearity, calibration curves, LODs, repeatability (% RSD) and reproducibility (%RSD) of the migration time, peak height and peak area, sensitivity enhancement factors (SEF) for standard samples, and recoveries for standard and river water samples.

Compounds	Linearity (ng/mL)	Calibration graphs			Repeatability (%RSD)(n=3) (10 ng/mL)			Reproducibility (%RSD)(n=5) (10 ng/mL)			SEF [a,b]		Recoveries (%)	
		Calibration curve	R^2	LOD (ng/mL)	Migration time	Peak height	Peak area	Migration time	Peak height	Peak area	SEF_{area}	SEF_{height}	5 ng/mL	25 ng/mL
Standard samples														
Bezafibrate	1-25	y= 3.16x + 4.58	0.996	0.25	1.5	0.7	1.2	4.5	6.5	5.1	14000	5600	97	100
Piroxicam	0.5-25	y= 3.27x + 7.60	0.998	0.12	1.6	1.6	2.1	4.9	2.8	4.0	13500	5900	90	82
Diclofenac Sodium	0.5-25	y= 4.22x + 9.59	0.998	0.10	1.7	2.1	0.9	5.0	3.1	2.9	12500	5800	108	102
Naproxen	0.25-25	y= 12.8x + 14.1	0.997	0.06	1.7	1.0	1.5	5.2	2.8	3.9	12500	5900	116	98
Clofibric acid	2.5-25	y= 3.1x + 2.82	0.998	0.5	1.8	1.7	2.4	5.4	2.3	5.3	11500	4700	97	82
River water samples														
Bezafibrate	2.5-25	y= 4.61x + 1.09	0.996	0.42	2.5	0.9	2.3	4.5	5.9	6.3	-	-	94	89
Piroxicam	1-25	y= 9.26x - 3.00	0.997	0.28	2.6	2.7	2.2	5.2	4.0	6.8	-	-	107	103
Diclofenac Sodium	1-25	y= 9.46x + 3.86	0.998	0.27	2.7	3.7	3.2	5.3	5.8	6.7	-	-	73	79
Naproxen	1-25	y= 14.8x + 5.44	0.998	0.19	2.8	2.6	2.0	5.5	6.3	5.3	-	-	82	82
Clofibric acid	5-25	y= 3.65x – 1.48	0.998	1.0	2.9	5.3	2.9	5.7	7.1	6.9	-	-	83	90

[a] Sensitivity enhancement factor for standard samples: SEF_{area} = dilution factor × ($area_{in\text{-}line}/area_{normal\ hydrodynamic\ injection}$);
SEF_{Height} = dilution factor × ($height_{in\text{-}line}/height_{normal\ hydrodynamic\ injection}$).

[b] Sensitivity enhancement factors for river water samples were not evaluated.

compounds provides adequate linerity, repeatability, and reproducibility, and the great potential of the method is observed by the high sensitivity enrichment factors obtained. The LODs obtained in the present study are lower to the ones obtained when several NSAIDs were determined in standard solutions by using an different preconcentration techniques in CZE as large-volume sample stacking using the EOF pump (LVSEP) [34], CEC as field-amplified sample stacking (FASS) [35], MEKC as stacking with reverse migrating micelles (SRMM), stacking with reverse migrating micelles - anion selective exhaustive injection (SRMM-ASEI) and field-enhanced sample injection with reverse migrating micelles (FESI-RMM) [36], or MEEKC as reversed electrode polarity stacking mode (REPSM) [37]. The reason is related with the larger volume of sample injected that allows the in-line SPE system. The LODs were also similar to the obtained with a more recently appeared electrophoretic preconcentration technique, which is electrokinetic supercharging (EKS) [38,39], since this combines the benefits of ITP and an electrokinetic injection of the sample, allowing a great amount of analytes to be introduced into the capillary. In the work from Macià *et al.* [18] naproxen was determined by an in-line SPE-CE method, in which the SPE sorbent (C18) was kept in place by using frits, and they obtained a LOD for standard solutions slightly higher than the obtained in this work.

3.4. Application

The optimized method was tested for the analysis of a group of acidic pharmaceutical compounds in river water samples. We carried out a direct injection of the sample, after adjusting the pH, in the same way that it was carried out for standard samples. As it has been mentioned before, with the river water samples the clean-up step for the SPE process has to be deeply considered, since in this case the complexity of the matrix is higher and this can play an important role. Different rinsing times were tested, from 3 to 6 min, as we did for standard samples, and we observed that a rinsing time of 3 min is enough to displace the sample solvent and to condition again the capillary, without observing losses in the recoveries.

Figure 4A shows the electropherogram obtained from the direct injection of the blank of the river water, after adjusting its pH at 3, to verify the absence of peaks at the same migration times than those of the analytes, and Figure 4B shows the electropherogram obtained from the direct injection of the river water spiked with a concentration of 5 ng/mL of the pharmaceutical compounds.

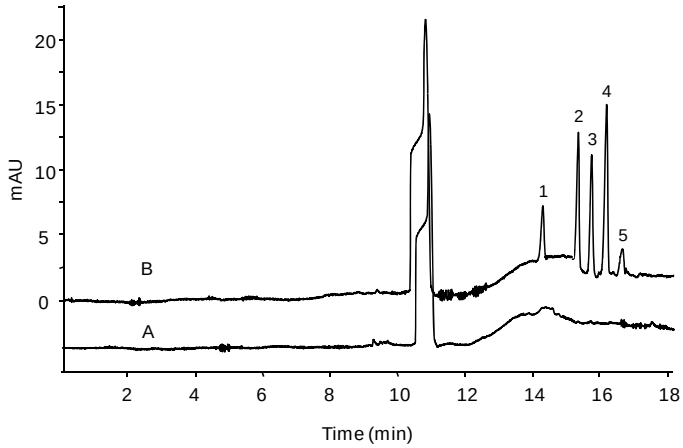

Figure 4. Electropherogram obtained under the optimum in-line SPE-CE conditions after analyzing (A) a blank river water sample injected at 930 mbar for 60 min. (B) a river water sample spiked with the analytes at a concentration of 5 ng/ml and injected at 930 mbar for 60 min. For peak assignment, see Figure 3. Other experimental conditions are reported in the text.

Table 1 shows the linearity, limits of detection (LODs), repeatability and reproducibility when river water samples at pH 3, spiked with the acidic drugs at different concentrations were analysed under the optimal in-line SPE-CE conditions. As it can be observed, the linear range in the calibration curve was 2.5-25 ng/mL for bezafibrate, 1-25 ng/mL for piroxicam, diclofenac sodium and naproxen, and 5-25 ng/mL for clofibric acid, with each coefficient of determination (R^2) being greater than 0.996. The limits of detection (LODs, S/N = 3) for the five analytes ranged from 0.19 to 1.00 ng/mL. The reproducibility and the repeatability, expressed as % RSDs, were evaluated in terms of migration time, peak height and peak area. Again, these values were evaluated at a concentration of 10 ng/ml on three different days (n=3) after the injection of each solution each day by performing three replicate analysis. Relative recoveries were also evaluated at two concentration levels, 5 ng/mL and 25 ng/mL, as is reported in the Table.

The results obtained for this group of pharmaceutical compounds when river water samples were analysed were compared to the obtained previously by other authors who used an off-line SPE process followed by different preconcentration approaches based on sample stacking techniques, CZE-MS, or in-line SPE-CE. In those studies, NSAIDs can be detected at a concentration range from 0.05 to 1.6 ng/mL by MEKC with SRMM, SRMM-ASEI and FESI-RMM [36], and from 0.1 to 0.250 ng/mL by MEEKC with REPSM when

mineral water samples were analysed [40], 0.1 ng/mL by CZE-MS for surface water samples [30], and 0.01 ng/mL for tap water samples by an in-line SPE-CE method, in which the SPE sorbent (C18) was kept in place by using frits, [18]. In all these previous studies, as we mentioned before, SPE was performed off-line before the electrophoretic method and in general, the preconcentration factors for this off-line pretreatment was between 100-fold and 1000-fold. Taking into account those preconcentration factors we can conclude that the LODs achieved by the in-line SPE-CE method developed in the present study which involves the direct injection of the river water sample, ranging from 1.19 to 1.00 ng/mL, are comparable to the ones reported previously in the bibliography for the same group of analytes which use a more exhaustive pretreatment of the sample.

We can also highlight that our results are in accordance with those obtained by other authors, who have also used an in-line SPE-CE method for the preconcentration and separation of different organic contaminants in environmental water samples [15,18,19, 24,26].

As it was mentioned in the introduction section, the concentrations of these compounds in surface waters are typically in the range of several tens to hundreds of ng/L so, in our case, it will be necessary a pretreatment to preconcentrate the sample before its injection in the in-line SPE-CE system. Further studies to decrease the limits of detection will be focused on the combination of the developed methodology with a previous preconcentration strategy, such as solid-phase extraction.

4. CONCLUSIONS

This paper demonstrates the great potential of the in-line SPE-CE method, using Oasis HLB as a sorbent, for the preconcentration and separation of a group of pharmaceutical compounds. The suitability of the methodology for the analysis of environmental waters was also tested with fortified river water samples. Under the optimized conditions, the sensitivity enrichment factors for these compounds in standard samples fell within the range 11500-14000 for peak area and 4700-5900 for peak height. The developed method allows the preconcentration and separation of the compounds in river water samples with a total analysis time of 90 minutes and obtaining LODs ranging between 0.19 and 1.0 ng/mL. The results obtained for this group of compounds in river water samples with a direct injection of the sample are comparable to those reported in the bibliography, in which other on-column preconcentration CE

techniques have been used. Furthermore, it is remarkable than they use an exhaustive off-line sample pretreatment and preconcentration.

It has been the first time that an in-line SPE-CE method has been developed to determine a group of acidic pharmaceutical compounds in river water samples. This method has been proposed as a satisfactory alternative to other preconcentration methods in CE, due to its avantatges as is cheaper, faster, not very tedious, and allows the same level of automation of conventional CE.

Acknowledgements

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PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
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3.2.2. Determination of UV-filters in river water samples by in-line solid-phase extraction-capillary electrophoresis-mass spectrometry

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DETERMINATION OF UV-FILTERS IN RIVER WATER SAMPLES BY IN-LINE SOLID-PHASE EXTRACTION-CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

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Abstract

The use of solid-phase extraction coupled in-line to capillary electrophoresis using electrospray mass spectrometry detection (in-line SPE-CE-ESI-MS) was investigated for the preconcentration and separation of four UV-filters: benzophenone-3 (BP-3), 2,2-dihydroxy-4-methoxybenzophenone (DHMB), 2,4-dihydroxybenzophenone (DHB) and 2-phenylbenzimidazole sulphonic acid (PMDSA). Firstly, a CE-ESI-MS method was developed and validated using standard samples, obtaining LODs between 0.06 µg/mL and 0.40 µg/mL. For the in-line SPE-CE-ESI-MS method, three different sorbents were evaluated and compared: Oasis HLB, Oasis MCX and Oasis MAX. For each sorbent, the main parameters affecting the preconcentration performance, such as sample pH, volume and composition of the elution plug, and sample injection time were studied. The Oasis MCX sorbent showed the best performance and was used to validate the in-line SPE-CE-ESI-MS methodology. The LODs reached for standard samples were in the range between 0.01 and 0.05 ng/mL with good reproducibility and the developed strategy provided sensitivity enhancement factors between 3400-fold and 34000-fold. The applicability of the developed methodology was demonstrated by the analysis of UV-filters in river water samples.

Keywords: In-line solid-phase extraction; capillary electrophoresis; UV-filters; river water samples.

1. INTRODUCTION

UV-filters are compounds that prevent damage on the skin and either block or reflect UV radiation. These compounds are present in personal hygiene products such as toothpaste, soaps and sunscreen cosmetics and they are included in the group of emerging contaminants known as personal care products (PCPs). These compounds not only reach the aquatic environment via wastewater treatment plants, like other contaminants, but also directly from recreational activities, such as sunbathing and swimming in seas, lakes and rivers [1,2]. Several studies have indicated that these substances are persistent in the environment, bioactive and have the potential for bioaccumulation, causing endocrine disruption and developmental toxicity. Therefore, monitoring these compounds in the aquatic environment has gained particular interest in recent years [2-10].

Different instrumental techniques have been used to determine UV-filters in environmental water samples. In this respect, these compounds have been determined by high-performance liquid chromatography (HPLC) with UV or mass spectrometry detection (MS) [11,12], HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) [13-18], gas chromatography-mass spectrometry (GC-MS) [19-22] and gas chromatography-tandem mass spectrometry (GC-MS/MS) [23-25]. Furthermore, concentration and/or clean-up techniques are employed in order to improve the sensitivity and limits of detection and/or to eliminate some potentially interfering compounds, since the usual levels of these compounds found in environmental waters are in the pg/mL range [1,2].

Capillary electrophoresis (CE) represents a very interesting alternative to chromatographic techniques due to the range of advantages it offers including simplicity, high separation efficiency, speed and low solvent consumption. This technique has been used to determine UV-filters compounds in PCPs [26-31], since they are found in concentration levels that could be achieved by CE without any previous preconcentration step. However, determination of UV-filters by CE in environmental waters samples has not been studied so far. Recently, Deng *et al.* [32] have analysed benzophenones in environmental water samples after topical skin application using dispersive liquid-liquid microextraction (DLLME) and micellar electrokinetic capillary chromatography (MEKC).

To improve the sensitivity of CE and expand its use for trace analysis, several approaches have been reported, such as the use of more sensitive detectors or preconcentration strategies [33-35]. In the case of the

detection system to improve sensitivity MS has been the most usual choice. Advantages of MS detection include improvement of detection sensitivity as well as the ability both to determine the exact mass of analytes and to provide structural information including the possibility of identifying and determining co-migrating species in overlapping peaks [33]. Despite the high selectivity and the improvement of the sensitivity achieved by the CE-ESI-MS systems, the small sample injection volumes remain a limitation to reach LODs low enough to measure the low concentrations at which UV-filters compounds are usually found in the aquatic environment.

To improve its sensitivity, CE can be combined with preconcentration techniques [34,35] . One of the most widely used approaches is based on the use of a chromatographic preconcentration technique such as a solid-phase extraction (SPE). SPE can be combined with CE in off-line, at-line, in-line or on-line modes [35]. In the in-line SPE-CE systems, the SPE sorbent or analyte concentrator (AC) is fully integrated into the CE configuration and the separation voltage is applied across the SPE material. The main advantages of this coupling are that it is easily automated, requires a low volume of organic solvent consumption through the process, needs a small quantity of sorbent material for the construction of the SPE device and is capable of analysing the complete eluate by CE [35]. In general, the sorbents most commonly used to build the in-line SPE-CE systems are reversed-phase sorbents (silica modified with C18 chains or hydrophilic polymer-based sorbents such as Oasis HLB) and mixed-mode sorbents, such as Oasis MCX, which is based on the same skeleton as Oasis HLB with additional strong cation-exchange moieties. For example, C18 sorbent has been used to determine peptides in human plasma, as well as ochrotoxin, naproxen, ceftiofur and chlorophenols in river water samples [36-39]. Oasis HLB has been used to preconcentrate a group of sulphonamides, drugs of abuse and NSAIDs in environmental water samples [40-42]. Oasis MCX sorbent has been used to preconcentrate 3-nitrotyrosine in rat urine and quinolone antibiotics in chicken muscle [43,44].

The main aim of this work has been to develop an in-line SPE-CE-ESI-MS method for the preconcentration and separation of UV-filter compounds. Firstly, the appropriate CE-ESI-MS conditions were optimized and the method was validated for standard compounds. For the optimization of the in-line SPE-CE-ESI-MS methodology, three different sorbents were evaluated and compared: Oasis HLB, a hydrophilic polymeric sorbent, Oasis MCX and Oasis MAX, which have the Oasis HLB

skeleton with additional cation-exchange and anion-exchange moieties, respectively. Quality parameters, such as repeatability, reproducibility, LODs and linearity, were also determined for the developed methodology. To show the applicability of the method for the analysis of environmental water samples, river water samples fortified with the UV-filters have been analysed applying additionally off-line SPE as a preconcentration and a clean-up step.

2. MATERIAL AND METHODS

2.1. Standards and reagents

Standards of the four UV-filters: benzophenone-3 (BP-3), 2,2-dihydroxy-4-methoxybenzophenone (DHMB), 2,4-dihydroxybenzophenone (DHB), and 2-phenylbenzimidazole sulphonic acid (PMDSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide (98%), ammonium hydrogen carbonate (p.a.), ammonium acetate, acetonitrile, acetone, methanol, 2-propanol, dichloromethane, formic acid, and ammonia solution (30 %), were supplied by Carl Roth GmbH (Karlsruhe, Germany). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Oasis HLB, Oasis MCX and Oasis MAX sorbents, with an average particle size of 60 µm, was supplied by Waters (Milford, MA, USA).

A stock standard solution for each UV-filter compound at 1000 mg/L was prepared in methanol. A mixed standard solution of the four compounds was prepared weekly at a concentration of 100 mg/L in methanol. The working standard solutions were prepared daily by diluting appropriate volumes of the standard solutions with Milli-Q water. All solutions were stored in a dark-glass flask at 4 °C. The background electrolyte (BGE) solution was prepared daily, and all buffers and working solutions were sonicated before use.

2.2. Instrumentation

CE analyses were performed on an HP 7100 CE system (Agilent Technologies, Waldbronn, Germany) coupled to a micrOTOFQ (Bruker Daltonik GmbH, Bremen, Germany), a quadrupole time-of-flight mass spectrometer (QTOF-MS). ESI ionization was applied using a sheath liquid interface from Agilent Technologies (Waldbronn, Germany). For data processing, the software Data Analysis version 4.0 (Bruker Daltonik) was used. Bare fused-silica capillaries with 50 and 150 µm I.D. were purchased from Polymicro (BGB Analytik Vertrieb, Schlossboeckelheim, Germany). All pH measurements were per-

formed with a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain).

2.3. CE-ESI-MS

Bare fused-silica capillaries with a total length of 75 cm and inner diameter (I.D.) of 50 µm were used for the electrophoretic separations. All capillary rinses were performed at 930 mbar. The BGE consisted of an aqueous solution of 20 mM of ammonium carbonate adjusted to pH 9.5 with 3 % ammonium hydroxide. Before the first use, the capillaries were conditioned by flushing with 1 M NaOH for 15 min, then with water for 20 min, and finally with the BGE solution for 30 min. At the beginning of each day, the capillaries were conditioned for 5 min with 0.1 M NaOH, for 10 min with water and finally with BGE for 15 min. Between runs, the capillaries were conditioned by flushing with 0.1 M NaOH for 2 min, then with water for 4 min, and finally with the BGE solution for 5 min. Samples were hydrodynamically injected at 50 mbar for 5 s. Analyses were carried out at 25 °C under normal polarity. A separation voltage of 20 kV was employed for the electrophoretic separations. The sheath liquid used was water–isopropanol (1:1, v/v) at a flow rate of 240 µL/h. The ESI source was operated in the negative mode with a positive MS inlet (5 kV) and a grounded sprayer. Nebulizer gas (N_2) pressure was set to 0.2 bar. The flow rate of the drying gas (N_2) was 4.0 L/min at a temperature of 170 °C. The mass spectrometer was operated in a mass range between 50-650 m/z at a repetition rate of 1 s. The instrument was calibrated by injection of a calibrant solution of sodium formate clusters every day before the first use.

2.4. In-line SPE-CE-ESI-MS

2.4.1. SPE-CE concentrator devices

AC which contained the SPE sorbent was made in-house according to a method described in a previous work [41]. Briefly, firstly a 75 cm (50 µm I.D. × 360 µm O.D.) of fused-silica capillary was conditioned (1 M NaOH for 15 min, water for 20 min, BGE for 30 min, methanol 5 min, and air for 15 min), and then, it was then cut into two pieces of 7.5 cm (inlet capillary) and 67.5 cm (separation capillary) in order to insert the AC between them. The 2 mm long AC (150 µm I.D. × 360 µm O.D.) was filled with Oasis MCX sorbent (60 µm of average size). The entire process of fabricating the concentrator was monitored under a microscope. Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow restriction before the analysis.

2.4.2 In-line SPE-CE-ESI-MS methodology

All in-line SPE-CE capillaries were conditioned with BGE for 15 min at the beginning of each day. Before each injection, the capillary was first conditioned by consecutives flushes of ACN for 2 min, and Milli-Q water at pH 3 for 3 min at 930 mbar. The sample adjusted at pH 3 with diluted formic acid was then loaded at 930 mbar for 15 min. The next step was a sample clean-up with BGE solution by applying 930 mbar for 3 min, in order to eliminate non-retained molecules and equilibrate the capillary before the elution and the electrophoretic separation. Afterwards, the retained analytes were eluted by injecting a plug of ACN, using a pressure of 50 mbar for 30 s (30 nL) and followed by an injection of BGE at 50 mbar for 200 s to push the elution plug through the concentrator (ca. 8 cm using Hagen-Poiseuille equation). Finally, a voltage of 18 kV was applied to perform the electrophoretic separation. After each injection the capillary was rinsed for 2 min with ACN and 2 min with water, in order to avoid any possible carry-over problems between consecutive injections.

2.5. Water sample pretreatment

River water samples from the Danube River (Germany) were provided by the water treatment plant, Zweckverband Landeswasserversorgung, from Danube River (Germany). Samples were collected by using pre-cleaned amber glass bottles and, prior to the extractions, they were adjusted to pH 3 with 37 % HCl. The off-line SPE applied to river water samples was performed with 60 mg Oasis MCX [14,15]. The off-line SPE was performed following the SPE protocol used for the determination of a group of PPCPs in river waters [15]. The SPE cartridges were conditioned with 2 mL of methanol and equilibrated with 2 mL of 2 % HCOOH (pH 2.1) at a flow rate of 3 mL/min. One litre of acidified and filtered water sample was passed through the cartridge at a flow rate of 4 mL/min. The cartridges were subsequently washed with 2 mL of 2% HCOOH, at a flow rate of 3 mL/min. After drying, the compounds were eluted with 2 mL of MeOH and 1 mL of 5 % NH_4OH in MeOH at a flow rate of 1 mL/min. Extracts were reduced to dryness under a gentle flow of N_2 gas. The extracts obtained from the off-line SPE were reconstituted with 5 mL of Milli-Q water at pH 3.0 adjusted with diluted formic acid, and filtered through a 0.22 µm nylon syringe filter.

3. RESULTS AND DISCUSSION

3.1. Separation by CE-ESI-MS

Before performing the study of the CE separation of the compounds, the MS parameters were optimized. A standard solution of each compound was used to select exact mass. The four analytes were detected as $[M-H]^-$, specifically BP-3 at m/z 227.072, DHMB at m/z 243.067, PMDSA at m/z 273.035 and DHB at m/z 213.056. Other conditions for the TOF-MS instrument are given in Section 2.3.

According to the pKa values of the compounds (see Table 1), a basic buffer is necessary for the separation of the compounds by CZE. Furthermore, taking into account the MS detector, a volatile buffer of low conductivity (i.e. electric current below 50 µA) is required to facilitate electrospray ionization and to avoid contamination of the MS. Hence, two basic and volatile BGEs based on ammonium hydrogen carbonate and ammonium acetate were tested. To find out the optimum BGE, the variation of the BGE concentration (10-50 mM), the BGE pH (pH from 8 to 10 adjusted with 3 % ammonium hydroxide) and the separation voltage (10 to 25 kV) were examined for each buffer. In general, the profiles obtained using the ammonium hydrogen carbonate buffer were better (in terms of resolution and peak efficiency) than those obtained with the buffer based on ammonium acetate. The best separation was obtained with 30 mM of ammonium hydrogen carbonate buffer at pH 9.5 adjusted with 3 % ammonium hydroxide. The separation voltage selected was 20 kV. Higher voltages increased the intensity of the electrical current up to values higher than 50 µA which can produce problems with the MS interface and with the in-line SPE system. Figure 1A shows the CE-ESI-MS extracted ion electropherograms (EIE) for a standard sample containing the four UV-filters at a concentration of 1 µg/mL. As can be observed, the four compounds were separated in less than 7 min.

3.2. Validation of the CE-ESI-MS analytical method

Under the optimal conditions, the CE-ESI-MS methodology was evaluated in terms of linearity, limits of detection (LODs), repeatability and reproducebility (Table 2). The quality parameters were established by measuring the peak area and migration times in the EIE. The LODs, (S/N = 3) for the four analytes ranged from 0.06 to 0.40 µg/mL. The repeatability, expressed in terms of relative standard deviation (% RSDs), was evaluated by ten replicate experiments (n=10) at a concentration of 10 µg/mL, and the values were

Table 1. Analyte abbreviations, structures and analytically relevant data of UV filters.

Abbreviation	Name	CAS nº	Empirical formula	Structure	pK_a
BP-3	Benzophenone	131-57-7	$C_{14}H_{12}O_3$		7.6
DHB	2,4-Dhydroxy benzophenone	131-56-6	$C_{13}H_{10}O_3$		7.7
DHMB	2,2-Dhydroxy-4-methoxy benzophenone	131-53-3	$C_{14}H_{12}O_4$		7.1
PMDSA	2,-Phenylbenzimidazole-5-sulphonic acid	27503-81-7	$C_{13}H_9O_3N_2S_1$		4.45

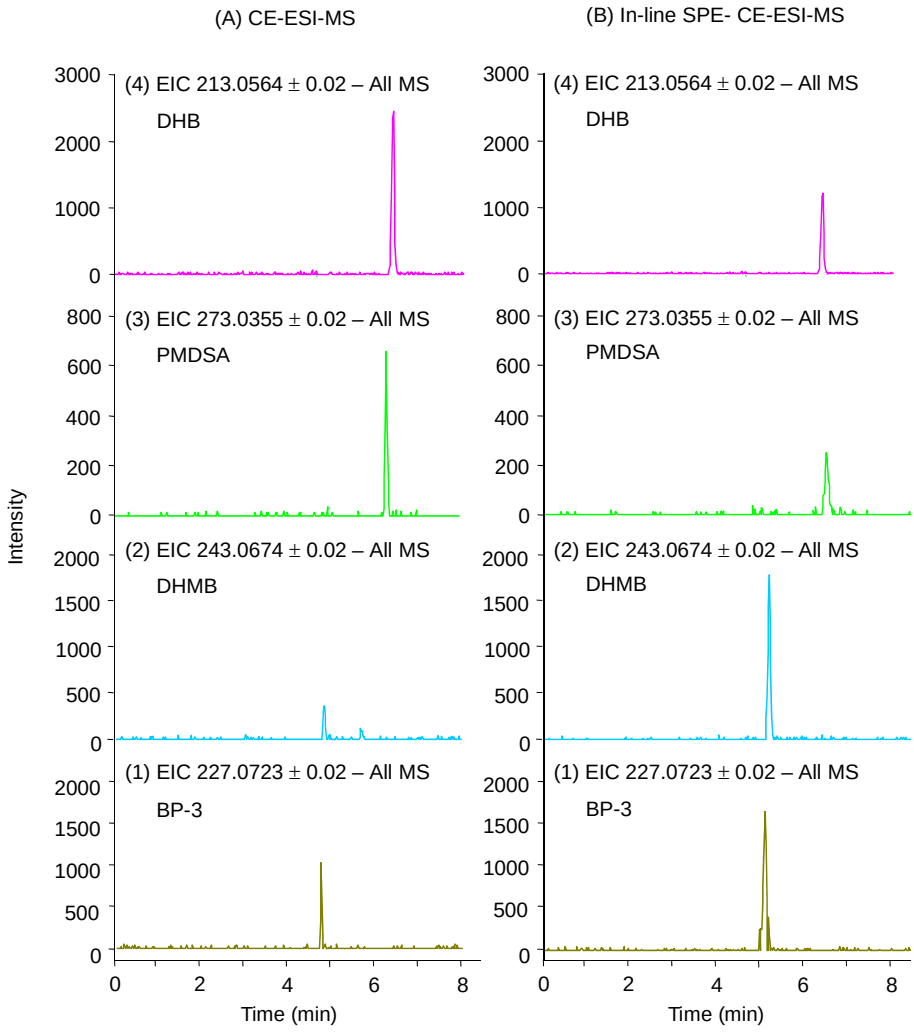

Figure 1. Extracted ion electropherograms (EIE) of: (A) Standard solution containing the compounds at a concentration of 1 µg/mL, injected hydrodynamically at 50 mbar 5 s, analysed by CE-ESI-MS. (B) Standard solution containing the compounds at a concentration of 0.1 ng/mL, injected hydrodynamically at 930 mbar 15 min, analysed by in-line SPE-CE-ESI-MS. BGE: 20 mM ammonium hydrogen carbonate pH 9.5, separation voltage 18 kV.

lower than 1.5 % for migration time and 8.7 % for peak area. The day-to-day reproducibility, expressed in terms of RSDs, was calculated by performing five replicate determinations of a standard solution at a concentration of 10 µg/mL on five different days (n=5). The values were lower than 3.1 % for migration time and 11.7 % for peak area.

Electrophoresis (submitted)

Table 2. Linearity, calibration curves, LODs, repeatability and reproducibility (%RSD) for migration time and peak height for standard samples by CE-ESI-MS

Compounds	$[M-H]^-$ m/z	Linearity (µg/mL)	Calibration graphs			Repeatability (% RSD) (n=10) (10 µg/mL)		Reproducibility (% RSD) (n=5) (10 µg/mL)	
			Calibration curve	R^2	LOD (µg/mL)	Migration time	Peak area	Migration time	Peak height
BP-3	227	0.3-20	y= 2289.5x - 287.3	0.999	0.15	1.5	6.5	2.6	10.8
DHMB	243	0.9-20	y= 1152.9x - 77.3	0.999	0.40	1.4	8.7	2.8	11.7
PMDSA	273	0.6-20	y= 3187x - 219.4	0.999	0.17	1.1	5.4	3.1	9.4
DHB	213	0.2-20	y= 15910x - 22261.7	0.999	0.06	1.1	6.4	2.4	8.5

Table 3. Linearity, calibration curves, LODs, repeatability and reproducibility (%RSD) of peak height, and sensitivity enhancement factors (SEF) for standard samples by in-line SPE-CE-ESI-MS

Compounds	$[M-H]^-$ m/z	Linearity (ng/mL)	Calibration graphs			Repeatability (% RSD) (n=10)		Reproducibility (% RSD) (n=5) AC[a]		SEF_{LODs} [b]
			Calibration curve	R^2	LOD (ng/mL)	1 (ng/mL)	10 (ng/mL)	1 (ng/mL)	10 (ng/mL)	
BP-3	227	0.05-50	y= 58070x + 4635.3	0.999	0.01	12.5	10.4	17.8	19.7	15000
DHMB	243	0.05-50	y= 19683x + 5954.3	0.997	0.01	15.7	13.2	15.7	16.9	34000
PMDSA	273	0.1-50	y= 2300x + 1012.5	0.998	0.05	14.4	12.8	18.4	19.4	3400
DHB	213	0.05-50	y= 38794x + 9271.6	0.999	0.01	18.7	14.7	19.4	21.4	6000

[a] Five different analyte concentrators (AC) (n=5).
[b] Sensitivity enhancement factor for standard samples: SEF_{LODs} = dilution factor × ($LODs_{CE\text{-}ESI\text{-}MS}/LODs_{in\text{-}line\ SPE\text{-}CE\text{-}ESI\text{-}MS}$).

3.3. Optimization of the in-line SPE-CE-ESI-MS method

For the optimization of the in-line SPE-CE-ESI-MS method, different sorbents were studied, in particular Oasis HLB, Oasis MCX and Oasis MAX. Off-line SPE extraction with Oasis HLB [13,16,23,25] and even Oasis MCX and Oasis MAX [14,15] followed by HPLC have been used to extract and preconcentrate PCPs compounds, including UV-filters. For each sorbent, the main parameters that affect the preconcentration factors were investigated: the sample pH, the elution solvent and its volume, and the sample loading time.

3.3.1. OASIS HLB

Oasis HLB is a polymeric sorbent with a polar group, with a hydrophilic-lipophilic-balance and reversed-phase interacttions. As a result, it is a universal sorbent for acidic, basic, and neutral compounds [13,16,23,25,45,46]. The sample pH was studied to ensure the most suitable conditions for retaining all the analytes. Taking into account the pKa values of this group of compounds (i.e. between 4.45 and 7.7), the influence of adjusting the sample at lower, similar and higher pH value than the pKa values was examined. Thus, sample pH values of pH 3.0, 5.0 and 9.5 were evaluated. For this study, a standard solution containing 100 ng/mL of each UV-filter was injected for 2 min at 930 mbar and then eluted by injecting a plug of ACN for 10 s at 50 mbar. The best results in terms of peak area were obtained at pH 3, at which they are retained by the strength of their hydrophobic interacttions with the sorbent. To study the elution step, different organic solvents reported in the literature were tested: MeOH, ethyl acetate: dichloromethane (DCM) (1:1), 5 mM ammonium hydrogen carbonate in MeOH, MeOH with 5% of NH_4OH, ACN, and acetone [13, 14,15,16,23,25,45,46]. In this case, the elution of the compounds was carried out by injecting a plug of elution solvent for 10 s at 50 mbar. Of all the organic solvents, ACN provided the best results in terms of peak area. Using ACN, the elution volume was then studied through a hydrodynamic injection from 10 to 40 s (10 to 40 nL) at 50 mbar. It was observed that the peak area values for the different compounds increased with the elution volume up to 30 s (30 nl). Finally, in order to load the maximum sample volume and establish the maximum capacity of the Oasis HLB sorbent, the sample injection time was optimized using a standard solution of 1 ng/mL injected at 930 mbar from 2 to 30 min with 30 s of ACN as elution plug. A linear relation of the peak area versus sample loading time was observed until

30 min, indicating that the breakthrough volume was not reached.

3.3.2. Oasis MAX

Oasis MAX is based on Oasis HLB with additional quaternary amines moieties and, thus, combines reversed-phase sorbent and strong anion-exchange interactions. Among the different parameters to be optimized, there are the sample conditions in order to assure the retention of the analytes in the sorbent. In order to exploit the ion exchange retention mechanism between the sorbent and the analytes, the sample should be adjusted to a high pH, so that the analytes, which are weak acidic, become ionized. Thus, the sample was adjusted at pH 9.5 with 3% ammonium hydroxide to ensure the deprotonation of the compounds. As elution solvents, MeOH with 2% of HCOOH and ACN with 2% of HCOOH were tested. In this way, the analytes become neutral and they are released from the sorbent. For this study, a standard solution containing the UV-filters at a concentration of 100 ng/mL was injected for 2 min at 930 mbar and then eluted by injecting a plug of solvent for 10 s at 50 mbar. When ACN containing 2 % of HCOOH was used, the results were better in terms of peak area. However, for PMDSA, no signal was obtained for any of the tested solvents. The effect of an increase of the elution solvent plug was therefore evaluated. A hydrodynamic injection of acidified ACN from 10 to 40s (10 to 40 nL) at 50 mbar was performed. Higher elution plugs of acidified ACN were not tested to avoid current instability and breakdown during the electrophoretic separation. It was observed that the peak area for the different compounds increased with the elution volume until 30 s (30 nl), except for the PMDSA which was still not eluted. This is probably due to the strong ionic interactions between the sulphonic group of this compound and the quaternary amine of the sorbent, even in the acidic conditions used for the elution. Finally, the sample loading time was studied, using a standard solution of 1 ng/mL injected at 930 mbar from 2 to 30 min and with an elution volume of 30 nL (30 s). A linear relation of the peak area versus loading time was observed up to 30 min, meaning that the breakthrough volume was not reached.

3.3.3. OASIS MCX

Oasis MCX is based on Oasis HLB with additional presence of sulphonic groups and, thus, combines reversed-phase sorbent and strong cation-exchange interactions. It is designed for the extraction of basic and neutral compounds. Basic compounds are retained on the sorbent through

cation-exchange interactions and acidic compounds are retained on the sorbent by means of reversed-phase interactions. The suitability of the MCX sorbent for the preconcentration of the studied compounds was evaluated by adjusting the sample pH to 3.0. ACN and MeOH were the elution solvents tested for standard solutions containning 100 ng/mL of each UV-filter and injected for 2 min at 930 mbar. The elution was performed by a plug of each elution solvent for 10 s at 50 mbar, and the best results in terms of peak area were provided by ACN. The elution plug volume was then optimized by modifying the injection time from 10 to 50 s. The results were the same as those obtained using the other sorbents, so an elution volume of 30 nL (30 s) was selected. The injection time of the sample was evaluated from 2 to 30 min. In this case, a linear relation of the peak area versus loading time was observed up to 30 min, which means that the breakthrough volume was not reached.

3.4. Comparison between the evaluated sorbents

Figure 2 shows the obtained results in terms of peak area for each sorbent studied at their optimal conditions. This figure shows that Oasis MAX generally provided a better preconcentration than the sorbent based on reversed-phase interactions, Oasis HLB, except for PMDSA. When Oasis MAX was used, the compounds were retained by ionic interactions and they were eluted by an acidic organic solvent (ACN with 2% of HCOOH), this elution solvent disrupted the ionic interactions in the case of BP-3, DHB and DHMB. On the other hand, PMDSA, which contains a sulphonic group (pka <1), still has an anionic charge under these conditions, so that the strong ionic interactions with the sorbent were not disrupted and the compound was not eluted. In the figure, it can be observed that the best preconcentration of the compounds was obtained with the Oasis MCX. Several articles reported greater recoveries for some UV-filters when off-line SPE with Oasis MCX was used than those provided with Oasis HLB [15,47]. However, this behaviour is rather surprising because BP-3, DHMB and DHB were in their neutral form, so the ionic interactions did not take place and just the reverse-phase interactions occurred, which should be similar to those in Oasis HLB. Consequently, a similar retention to the Oasis HLB sorbent would be expected. However, our results show that these compounds are better retained in the Oasis MCX sorbent. This may be explained by the enhanced hydrophobic and polar retention characteristics of the Oasis MCX compared to Oasis HLB with the

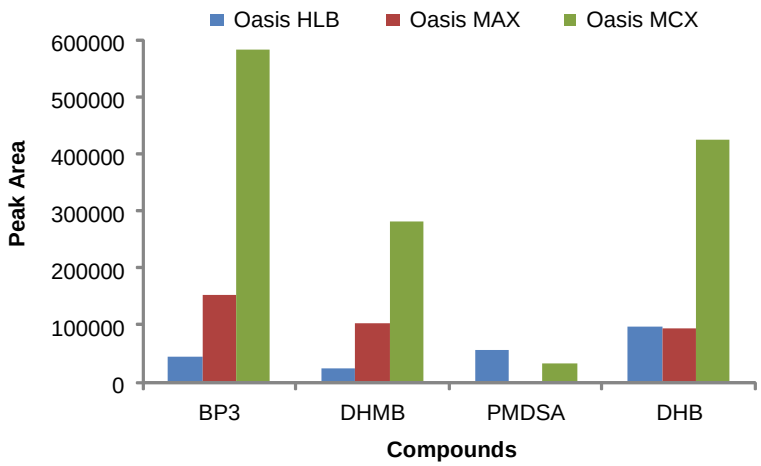

Figure 2. Profile showing the peak height obtained for each compound with the three sorbents (Oasis HLB, Oasis MAX, Oasis MCX). Sample: standard solution containing the compounds at a concentration of 10 ng/mL was injected by applying 930 mbar for 15 min; elution was carried out by injecting the elution plug at 50 mbar for 30 s. For other experimental conditions, see Figure 1.

same backbone structure as was previously observed by other authors [48]. As the best preconcentration factors were achieved with the mixed-mode cation-exchange sorbent, Oasis MCX, it was selected to validate the analytical method and to show the potential of in-line SPE system for analysing river water samples.

The optimal conditions for the in-line SPE(MCX)-CE-ESI-MS method were as follow: BGE composed of 30 mM ammonium hydrogen carbonate at pH 9.5; sample matrix composed of Milli-Q water at pH 3; the sample was injected hydrodynamically for 15 min at 930 mbar; and the elution solvent, ACN, was also injected hydrodynamically for 30 s at 50 mbar. Figure 1B shows the extracted ion electropherograms (EIE) obtained when a standard sample containing the UV-filters at 0.1 ng/mL was analysed under the optimal in-line SPE-CE-ESI-MS conditions. As can be observed, similar electrophoretic separations were obtained using CE-ESI-MS (Figure 1A) and in-line SPE-CE-ESI-MS (Figure 1B), and the presence of the in-line SPE did not have a significant effect on peak shape or separation resolution, although a slightly delay in migration times can be observed. Furthermore, by the comparison of Figure 1A and 1B, an improvement of sensitivity is achieved using the developed methodology based on in-line SPE-CE. After optimizing the experimental conditions for in-line SPE-CE-ESI-MS, limits of detection for a mixture of four UV-filters have been

lowered up to 34,000-fold compared to the values previously obtained by CE-ESI-MS.

3.5. Validation of the in-line SPE-CE-ESI-MS analytical method

The in-line SPE-CE-ESI-MS method was validated with standard samples for linearity, precision and limits of detection (LODs) (Table 3). Calibration curves were constructed for all the analytes from 0.05 to 50 ng/mL, except for PMDSA which had a range between 0.10 and 50 ng/mL. Excellent linearity was achieved in these concentration ranges for which correlation coefficients higher than 0.997 were calculated for all the compounds. The LODs (S/N = 3) for the four analytes ranged from 0.01 to 0.05 ng/mL. The repeatability was evaluated by ten replicate experiments (n=10) at two different concentration levels, 1 and 10 ng/mL, and the results obtained, expressed as relative standard deviation (% RSDs) were lower than 18.7 % for peak area. The day-to-day reproducibility was calculated by analysing a standard solution at two different concentrations, 1 and 10 ng/mL on five different days (n=5), using a different AC concentrator each day. The results obtained in this case were lower than 21.4 % for peak area. % RSD values were slightly higher for reproducibility than for repeatability and this may be related to the low variability due to the construction of the concentrators. It should be highlighted that repeatability and reproducibility values were relatively higher than those obtained by CE-ESI-MS. As far as we know, the LODs obtained in the present study are the lowest obtained by CE for the determination of UV-filters [26-32].

3.6. Application

As mentioned in the introduction, the concentration of these compounds in environmental waters is typically at pg/ml levels so, in our case, an additional pretreatment step is necessary to preconcentrate the sample before its injection in the in-line SPE-CE system. For instance, BP-3 has been found in river water samples at concentrations between 6 pg/mL and 28 pg/mL, using a previous preconcentration technique based on off-line SPE and subsequent determination by LC-MS [11]. It should be highlighted that this strategy is widely used before in-line SPE-CE, especially when handling with environmental samples. So we decided to carry out an off-line SPE step to preconcentrate the sample before the in-line SPE-CE-ESI-MS method. The experimental process has been described in the experimental section, and this off-line SPE approach provides an additional preconcen-

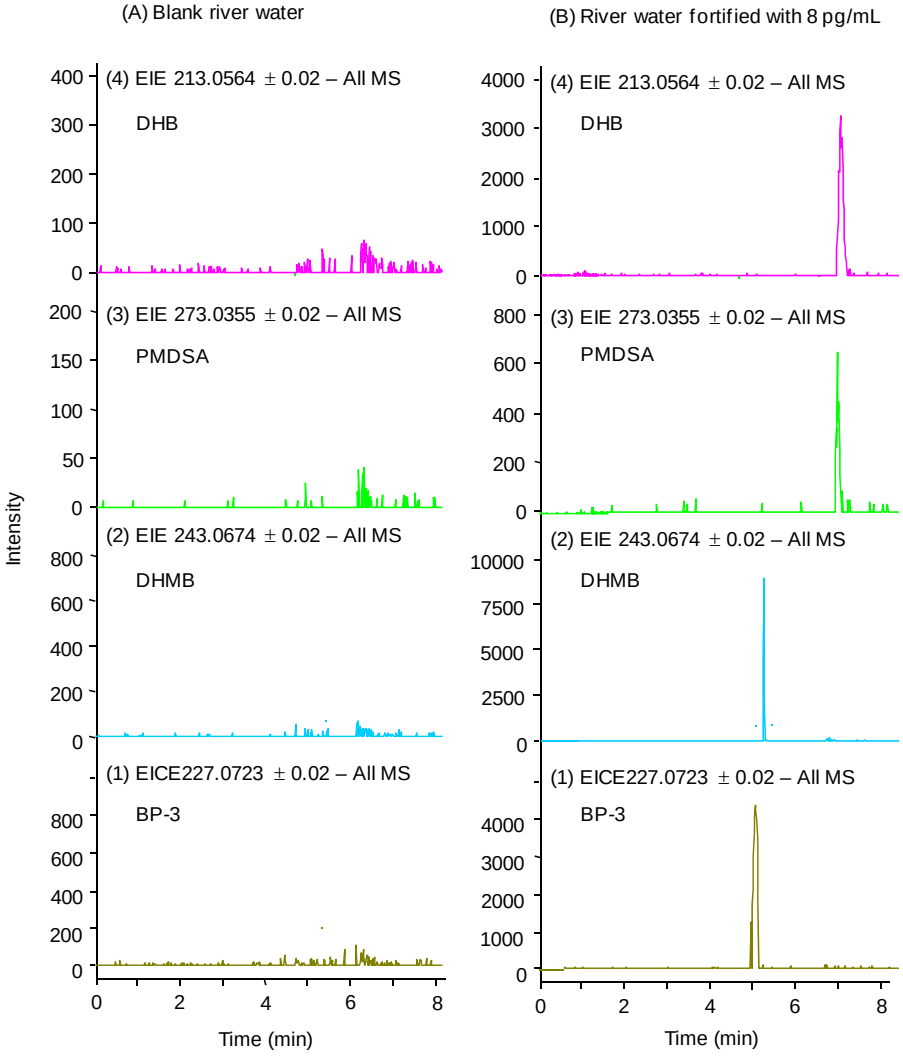

Figure 3. Extracted ion electropherograms (EIE) obtained with in-line SPE-CE-ESI-MS when analysing river water sample without (A) and with the addition of the studied analytes at 8 pg/mL (B). The experimental conditions are reported in the text.

tration factor of 200. To test the applicability of the developed overall method, real water samples were analysed. Figure 3 shows the electropherograms obtained for the off-line SPE followed by the in-line SPE-CE-ESI-MS analysis of a blank sample of river water (Figure 3A) and river water fortified with 8 pg/mL (Figure 3B). Clearly, all the four compounds can be detected at this low concentration in a river water sample. The combination between off-line SPE and in-line SPE provides a suitable approach for con-

centrating UV-filters in water samples at low pg/ml levels prior to CE-MS analysis, resulting in a strategy which reports similar results in terms of sensitivity as chromatographic methods [10,13-15].

4. CONCLUSIONS

An in-line SPE-CE-ESI-MS method has been developed for the determination of UV-filters, using Oasis MCX as a sorbent. The LODs for the four UV-filters have been lowered up to 3400-fold to 34000-fold, compared to the values obtained by CE-ESI-MS without in-line SPE. Since the proposed method, in-line SPE-CE-ESI-MS, is simple, selective, repeatable and effective, as demonstrated by the sensitivity and precision studies carried out, and it might be further extended to similar compounds. The method is useful for real applications, as shown by analysing fortified river water samples. The SPE injection (in-line SPE) notably increases sensitivity. However, additionnal off-line SPE is required to achieve the low pg/ml concentrations in environmental samples and to perform a clean-up of the sample. To the best of our knowledge, this strategy shows the first in-line SPE-CE-MS determination of these compounds.

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UNIVERSITAT ROVIRA I VIRGILI
PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
Irene Maijó Ferré
Dipòsit Legal: T. 1056-2012

3.2.3. Discussion of results

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The main goal of an in-line SPE-CE method is to optimize the extraction process by maximizing the recovery of the target analytes under conditions which provide efficient and reproducible CE separations. A variety of materials are used as sorbents in SPE for the extraction of compounds of interest. The choice of a sorbent is critical in SPE because the sorbent can influence parameters such as selectivity and capacity. A generic SPE-CE procedure consists of the following basic steps: conditioning, equilibration, sample loading, clean-up and conditioning step, elution, pushing step and electrophoretic separation [1-5].

Some of these steps only affect the SPE extraction, such as the conditioning of the SPE and the sample loading. However, the other steps affect both the SPE extraction and the electrophoretic separation. After the sample loading, a clean-up step with BGE is necessary, in order to remove unretained molecules as well as to condition the CE system in order to apply the separation voltage. Furthermore, this step is important to assure that there are no losses of the analytes from the sorbent. The volume of the elution solvent is a key parameter for the in-line SPE, as generally this volume is higher that the conventional hydrodynamic volumes introduced in CE. This can lead to lower peak efficiency and resolution, as well as problems with the stability of the current. Finally, the pushing step is necessary in order to push the elution solvent through the SPE before the CE separation.

In the first study, which focused on development of an in-line SPE-CE strategy to enhance sensitivity for the determination of pharmaceutical compounds (NSAIDs and lipid regulators) in river water, the optimal conditions for the electrophoretic separation were taken from a previous study carried out by our group [6]. The BGE was composed of 20 mM of ammonium acetate at pH 9.0 and the reverse-phase sorbent Oasis HLB was used to construct the in-line SPE-CE system. As the sample pH can greatly affect the retention of the compounds in the sorbent, this parameter was evaluated. The higher retention in the sorbent was obtained at an acidic pH value of pH 3.0, as at this pH, compounds are in their non-anionic form and they are retained by the strength of their hydrophobic interactions with the sorbent face. Methanol was selected as the elution solvent because it provides good results for this group of compounds through an off-line SPE with Oasis HLB [7]. The volume was evaluated within the range of 20 nL to 48 nL. The results showed that when the elution volume was higher than 34 nL, a loss of efficiency and resolution was observed. One of the advantages of in-line SPE-CE is the possibility of introducing a large amount of sample which enables higher pre-concentration factors to be achieved. Therefore, the sample loading time was optimized, achieving the higher sensitivity in a reasonable analysis time, when the

sample was injected for 60 min (46 µL). After the optimization, the developed in-line SPE-CE methodology was validated for standard samples. It was then applied to the determination of these compounds in river water samples. This methodology allowed the direct injection of the river water without any pretreatment or dilution. Table 3.2.1 shows the main results for the developed in-line SPE-CE system for both standard and river water samples.

The LODs achieved ranged between 0.06 ng mL^{-1} and 0.50 ng mL^{-1} for standard samples, which are similar than those obtained for Macià *et al.* [8] for the preconcentration of naproxen by an in-line SPE-CE method, using C18 as sorbent with frits to construct the AC, and also it should be highlight that in this study the AC device was longer than in our study (1 cm in front of 2mm). Generally, the chromatographic preconcentration technique developed in this section achieved LODs significantly lower than those reported by electrophoretic preconcentration techniques [9-15]. However, there are two studies developed by Dawod *et al*. [16, 17], based on EKS and CF-EKS, which reported LODs similar to those obtained in this study. Recently, that research group combined EKS with a positive pressure during injection (PA-EKS) to improve the preconcentration [18]. Under these conditions, LODs in the range of 0.0067 ng mL^{-1} and 0.0187 ng mL^{-1} were achieved, which are lower than those reported in this study.

River water samples were analysed without any pretreatment, obtaining electropherograms free of interferences. The LODs achieved for these samples, ranging from 0.19 ng mL^{-1} to 1.00 ng mL^{-1}, are comparable to those reported previously in the literature for the same group of analytes [9-15]. However, it should be highlighted that in those studies, a more exhaustive pretreatment of the sample is used. Further studies in the future to decrease the limits of detection will focus on the combination of the developed methodology with a previous preconcentration technique, such as SPE, because this pretreatment is necessary in order to determine these compounds at levels typically found in environmental waters.

The second study focused on the development of an in-line SPE-CE-MS strategy to enhance sensitivity for the determination of UV-filters compounds in river water. The electrophoretic separation of the compounds by CZE was studied, choosing two BGEs compatible with the MS detector, namely ammonium hydrogen carbonate and ammonium acetate. The best separation was obtained with a BGE composed of 30 mM of ammonium hydrogen carbonate at pH 9.5. For the optimization of the in-line SPE-CE preconcentration technique, three sorbents with different characteristics were selected: Oasis HLB, a hydrophilic polymeric sorbent; and Oasis MCX and Oasis MAX,

Table 3.2.1. Experimental conditions and results obtained for the in-line SPE-CE method for pharmaceutical compounds.

Standard samples

CE	Capillary	Sample volume	Elution volume	LODs	SEF_{LODs}
CZE-UV	50 µm I.D., 112.5 cm	3.4 nL (50 mbar 5 s)		523-1,960 ng mL^{-1}	
In-line SPE-CE-UV	50 µm I.D., 112.5 cm	46,000 nL (930 mbar 3,600 s)	34 nL (50 mbar 50 s)	0.06-0.50 ng mL^{-1}	3920-8717-fold

River water

CE	Capillary	Sample volume	Elution volume	LODs
In-line SPE-CE-UV	50 µm I.D., 112.5 cm	46,000 nL (930 mbar 3,600 s)	34 nL (50 mbar 50 s)	0.19-1.00 ng mL^{-1}

which have the Oasis HLB skeleton with cation-exchange and anion-exchange moieties, respectively. For the three sorbents, the main parameters affecting the in-line preconcentration were optimized and the results are summarized in Table 3.2.2. In the case of the Oasis HLB, the sample pH was studied, and the best results in terms of peak area were obtained at pH 3, at which the compounds are in their non-ionic form and are retained by the strength of their hydrophobic interactions with the sorbent face. In the case of Oasis MAX and Oasis MCX, the proper pH was selected for each sorbent based on their ionic exchange characteristics. For the Oasis MAX, the sample was adjusted to pH 9.5 to ensure the deprotonation of the compounds, in order to exploit the ion exchange mechanism. For the Oasis MCX, the sample was adjusted to pH 3, as acidic compounds are retained in the sorbent by means of reverse-phase interactions. For the optimization of the elution step, different organic solvents were tested for each sorbent and, in all cases, ACN provided the best results in terms of peak area. However, for the Oasis MAX, ACN was acidified with 2 % of HCOOH to neutralize the analytes and facilitate their elution from the sorbent. The volume of the elution solvent was then optimized and, for all the sorbents, an elution volume of 30 nL provided the best results without losses of efficiency and resolution. The sample was injected for 15 min (17 µL). This length of time was selected as a compromise between sensitivity and analysis time. Of the three sorbents, Oasis MCX provided the best preconcentration factors and so it was used to validate the in-line SPE-CE method with standard samples. River water samples fortified with 8 pg mL^{-1} were then analysed with a previous off-line SPE followed by the in-line SPE-CE method in order to show the applicability of the developed methodology. In this case, an additional off-line SPE was required to achieve the low pg mL^{-1} concentration in environmental samples and to perform a clean-up of the sample. Table 3.2.3 shows the main results for the in-line SPE-CE system for both standard and real samples.

Table 3.2.2. pH of the sample and elution solvent for each sorbent studied.

SPE sorbent	Sample pH	Elution solvent
HLB	3.0	ACN
MAX	9.5	ACN 2% HCOOH
MCX	3.0	ACN

The LODs achieved ranged between 0.01 ng mL^{-1} to 0.05 ng mL^{-1} for standard samples, which are very low, taking into account that only 17 µL of sample was loaded compared to the 47 µL of sample loaded in the study of the pharmaceutical compounds. It should be noted that, in this study, in addition to the in-line SPE-CE preconcentration technique, a more sensitive detector was also used, namely the MS detector. As far as we know, the LODs obtained in the present study are the lowest obtained by CE for the

Table 3.2.3. Experimental conditions and results obtained for the in-line SPE-CE method for UV-filter compounds.

Standard samples

CE	Capillary	Sample volume	Elution volume	LODs	SEF_{LODs}
CZE-MS	50 µm I.D., 75.0 cm	5.1 nL (50 mbar 5 s)		60-400 ng mL^{-1}	
In-line SPE-CE-MS	50 µm I.D., 75.0 cm	17,000 nL (930 mbar 3,600 s)	30 nL (50 mbar 30 s)	0.01-0.05 ng mL^{-1}	3,400-34,000-fold

River water

CE	Capillary	Additional pretreatment	Sample volume	LODs
In-line SPE-CE-MS	50 µm I.D., 75.0 cm	Off-line SPE	1000 mL	---

determination of UV-filters and the instrumental sensitivity is similar to those obtained by chromatographic methods [7].

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3.3. Comparison of different preconcentration strategies for the determination of parabens by capillary electrophoresis

UNIVERSITAT ROVIRA I VIRGILI
PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
Irene Maijó Ferré
Dipòsit Legal: T. 1056-2012

In the previous section, different electrophoretic preconcentration techniques, sweeping and ASEI-sweeping, and a chromatographic preconcentration technique, in-line SPE-CE, were studied for increasing the sensitivity of CE in order to be able to determine PPCPs in environmental samples. This final section of the Doctoral Thesis focuses on the comparison of different preconcentration techniques in order to increase the sensitivity of CE for determining a group of parabens.

The preconcentration techniques studied were the previous electrophoretic preconcentration technique of sweeping and the chromatographic preconcentration technique of in-line SPE-CE. In addition, LVSS and FASI were also studied, as these sample stacking techniques are commonly used in CZE due their simplicity. The main difference between the two techniques is the injection mode, which is hydrodynamic for LVSS and electrokinetic for FASI. For both techniques, the polarity was switched between the preconcentration step and the electrophoretic separation in order to remove the sample matrix from the capillary [1-2].

The parabens studied were methylparaben (MP), ethylparaben (EP), propylparaben (PP), isopropylparaben (IPP), butylparaben (BP), and benzylparaben (BzP). These compounds are widely used as bactericides and preservative agents in cosmetic, pharmaceutical and personal care products (e.g. bath gels, shampoos, deodorants, antiperspirants, creams and toothpastes), as well as in processed food and beverages, for inhibiting the development of microorganisms and therefore prolonging products' shelf lives [3,4]. These compounds are included in the group of emerging organic contaminants and, nowadays, the study of their presence in different kind of samples, including pharmaceutical, personal care products, food, biological samples and environmental samples has become a field of growing importance.

CE has mainly been applied in order to determine parabens in cosmetic, food and pharmaceutical compounds [5-8]. However, there are only two studies that focus on the determination of parabens in environmental samples. Blanco *et al*. [9, 10] developed a method based on the use of NACE and LVSS as a stacking technique for the quantification of parabens in wastewater samples, requiring the previous application of a preconcentration step by off-line SPE. The present study focused on the comparison of different preconcentration techniques in CE for the determination of these compounds, with the aim of applying them to real samples in future, such as biological and environmental samples.

The main parameters affecting the different preconcentration approaches were investigated in an attempt to compare the potential of these preconcentration methodologies for the determination of parabens at low concentration levels.

A paper discussing the results obtained in this study has been submitted for publication in *Electrophoresis* journal.

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3.3.1. Different strategies for the preconcentration and separation of parabens by capillary electrophoresis

UNIVERSITAT ROVIRA I VIRGILI
PRECONCENTRATION STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
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DIFFERENT STRATEGIES FOR THE PRECONCENTRATION AND SEPARATION OF PARABENS BY CAPILLARY ELECTROPHORESIS

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Abstract

Several strategies, namely large volume sample stacking (LVSS), field-amplified sample injection (FASI), sweeping, and in-line SPE-CE, were investigated for the simultaneous separation and preconcentration of a group of parabens. A background electrolyte (BGE) consisting of 20 mM sodium dihydrogenphosphate (pH 2.28) and 150 mM sodium dodecyl sulphate (SDS) with 15 % ACN was used for the separation and preconcentration of the compounds by sweeping, and a BGE consisting of 30 mM sodium borate (pH 9.5) was used for the separation and preconcentration of the compounds by LVSS, FASI, and in-line SPE-CE. Several factors affecting the preconcentration process were investigated in order to obtain the maximum enhancement of sensitivity. The limits of detection (LODs) obtained for parabens were in the range of 18-27 ng/mL, 3-4 ng/mL, 2 ng/mL, and 0.01-0.02 ng/mL, and the sensitivity evaluated in terms of peak areas was improved up to 28-, 109-, 232-, and 19000-fold for sweeping, LVSS, FASI, and in-line SPE-CE, respectively. These preconcentration techniques showed potential as good strategies for focusing parabens. The four methods were validated with standard samples to show the potential of these techniques for future applications in real samples, such as biological and environmental samples.

Keywords: Field-amplified sample injection (FASI); In-line SPE-CE; Large volume sample stacking (LVSS); Parabens; Sweeping.

1. INTRODUCTION

Parabens are one of the most widely used type of preservatives in cosmetic, pharmaceutical and personal care products (PPCPs), foods and beverages due to their broad spectrum of antimicrobial activity and their physicochemical properties. Some studies have demonstrated that exposure to

parabens may modulate or disrupt the endocrine system, exhibiting weak estrogenic and other hormone-related activity, and thus may have harmful consequences on human health and wildlife [1,2]. Due to their absorption from topical applications and ingestion from a diverse range of consumer products, parabens have been detected in human breast tissue, milk, urine and serum [3-5]. Furthermore, these compounds are continuously released into the environment through mainly urban wastewater, which has produced growing concern and interest in relation to their potential long-term effects not only on humans but also on other organisms [1,2,6-9]. Therefore, it is necessary to develop some highly selective and sensitive methods for the multi-constituent determination of parabens in different environmental samples. The majority of reported methods for the determination of these compounds are usually based on gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry detection (MS) [3-10]. In recent years, the development of electromigration techniques has meant that capillary electrophoresis (CE) has shown itself to be a very interesting alternative to chromatographic techniques due to its many advantages including simplicity, high separation efficiency, selectively, ease of instrumentation and low cost [11-24].

The European Economic Community (EEC) Directives, 76/768/EC and 95 /17EC allow the use of parabens in cosmetics with a maximum concentration for each one of 0.4% w/w and a total maximum concentration of 0.8% w/w, expressed as p-hydroxybenzoic acid (PHBA) [25]. Furthermore, even though they are not regulated, the maximum concentration of parabens in foods is 0.1% w/w, and in pharmaceuticals, paraben content seldom exceeds 1% w/w [26].

Capillary electrophoresis has been widely used to determine parabens in pharmaceuticals [11-13], personal care products [11,14-18] and food [19], since they are found in concentration levels that could be achieved by CE without any previous preconcentration step. Different CE modes, such as capillary zone electrophoresis (CZE) [11,12,19], micellar electrokinetic capillary chromatography (MEKC) [13-15,18], microemulsion electrokinetic chromatography (MEEKC) [14,16], and capillary electrochromatography (CEC) [17,20], have been used for this purpose.

Determination of parabens by CE in samples which contain those compounds at low concentration levels, such as environmental water samples, has not been widely studied [21,22]. In those studies, a previous pretreatment step, such as a solid-phase extraction (SPE), and a preconcentration tech-

nique applied to CE was necessary to achieve the low LODs at which these compounds can be found.

For significant improvement in the well-documented poor sensitivity of CE and an expansion of its use for trace analysis, several preconcentration strategies have been reported [27,28]. These strategies can be categorized into two groups based on the physical phenomena used to concentrate analytes. One group involves manipulating the electrophoretic velocity of the analytes may be classed as the electrophoretic techniques. This group includes techniques such sample stacking, isotachophoresis, pH-mediated stacking and sweeping [27]. The other group is those using chromatographic techniques, which involve partitioning into a stationary phase [28].

Among the electrophoretic preconcentration techniques, the sample stacking techniques are the most commonly used and include large volume sample stacking (LVSS) with a hydrodynamic injection of the sample, and field-amplified sample injection (FASI) with an electrokinetic injection of the sample. Sweeping is another electrophoretic preconcentration technique widely used in MEKC, which is useful for either ionic or neutral compounds.

LVSS was introduced by Chien and Burgi [29] and is based on the conductivity difference between the sample and buffer zones. This technique allows the hydrodynamic injection of a large volume of low conductivity sample, and analytes are stacked after passing through the boundary between the sample zone and the BGE, while the matrix is continuously removed from the capillary by the manipulation of the EOF. Before the analytes exit the capillary, matrix removal is stopped and separation begins. For LVSS with polarity switching, the point at which the polarity is switched is determined by monitoring the current profile, and is usually changed when the current reaches 95% of the value with the capillary completely filled with electrolyte [27]. There are two studies carried out by Blanco *et al*. [21,22] in which LVSS combined with nonaqueous capillary electrophoresis (NACE) was used for the determination of parabens in river waters, achieving LODs between 0.7 and 2.1 ng/mL. Another study was performed by He *et al* [24], and in this case, a combination of LVSS and MEKC was performed to determine parabens in cosmetic products with LODs around 50 ng/mL.

FASI is based on the conductivity difference between the sample and buffer zones but, in this case, the sample is loaded electrokinetically into the capillary. It has been reported that hydrodynamic injection of a plug of low

conductivity solvent, e.g. water, before electrokinetic sample injection establishes a high electric field at the injection point which made the stacking more effective [30]. When a sample prepared in a medium with lower conductivity than the BGE is injected, the ions stack at the boundary between the pre-injection plug zone of low conductivity and the BGE. Sensitivity enhancements factors (SEF) obtained by FASI (up to 2500-fold) are higher than those of LVSS (up to 500-fold), since the analytes are electrokinetically injected [27].

Sweeping was introduced by Quirino and Terabe in 1998 and is an effective on-line sample concentration technique in MEKC for charged and neutral compounds [31]. This strategy is based on the injection of a large volume of sample devoid of pseudostationary phase (PS), and subsequent picking and accumulation of the analytes into a narrow zone by the PS that penetrates the sample region. When no conductivity differences exist between sample matrix and the BGE, the concentration effect is only due to the interactions of the analytes with the PS. However, when the sample has a different conductivity than the BGE, the concentration effect is due to the sample stacking in MEKC and the chromatographic partitioning. In general, the enhancement factors ranged from 10 to 1500-fold, which allowed the analysis of different analytes in many samples [32].

Another interesting approach to increasing sensitivity in CE is using a chromatographic preconcentration step before the electrophoretic separation such as SPE. SPE has been combined with CE following different approaches. The simplest and the most commonly used way is with an in-line coupling between the SPE and the capillary [28]. Using this approach, the SPE sorbent or analyte concentrator (AC) is fully integrated into the CE configuration and the separation voltage is applied across the SPE material. This material can be retained by means of two frits to prevent the sorbent particles from entering and blocking the CE capillary, or without frits, using sorbent particles with sizes larger than the internal diameter of the separation capillary. In general, the sorbents most commonly used to build the in-line SPE-CE systems have been C18, Oasis HLB and Oasis MCX [33-38]. One of the main advantages of the in-line SPE-CE system is that it allows the injection of a large volume of sample, since capillary volume is not a limitation in this case as it is for electrophoretic preconcentration, so the SEF achieved is high.

There is a study carried out by Tennico *et al.* [23] in which an in-line SPE extraction using functionalized silica-coated iron oxide magnetic particles

for CE and microchip was developed for the automated extraction of several parabens and nonsteroidal anti-inflammatory drugs (NSAIDs).

The main aim of the present work was to show the potential of different preconcentration strategies based on different mechanisms (LVSS, FASI, sweeping, and in-line SPE-CZE) for the determination and preconcentration of a group of parabens. Firstly, the optimization of the separation of the compounds by CZE and MEKC was carried out. The main parameters affecting the different preconcentration approaches were then investigated. Finally, the optimized methodologies were validated with standard samples. To the best of our knowledge, this is the first attempt to compare these preconcentration methodologies for the determination of parabens at low concentration levels.

2. MATERIAL AND METHODS

2.1. Standards and reagents

Standards of the six parabens: methylparaben (MP), ethylparaben (EP), propylparaben (PP), isopropylparaben (IPP), butylparaben (BP), and benzylparaben (BzP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Orthophosphoric acid 85% (H_3PO_4), sodium borate ($Na_2B_4O_7$), sodium dihydrogenphosphate (NaH_2PO_4), and sodium dodecyl sulphate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide (98%) was from Panreac (Barcelona, Spain). Acetonitrile, acetone, methanol, 2-propanol, dichloromethane, formic acid, and ammonia solution 30%, were from SDS (Peypin, France). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). 500 mg Oasis HLB sorbent, with an average particle size of 60 µm, was supplied by Waters (Milford, MA, USA).

A stock standard solution of 1000 mg/L for each paraben was prepared in methanol. The mixture of all the compounds was prepared weekly at a concentration of 100 mg/L in methanol. The working standard solutions were prepared daily by diluting appropriate volumes of the standard solutions in the adequate solvent of each preconcentration technique. All solutions were stored in a dark-glass flask at 4 °C. BGE solution was prepared daily and all buffers and working solutions were sonicated and filtered through a 0.22 µm nylon syringe filter (Teknokroma, Barcelona, Spain) before use.

2.2. Instrumentation

CE analyses were performed on a Hewlett-Packard 3D CE instrument (Agilent Technologies, Waldbronn, Ger-

many) system equipped with a UV diode-array detector (DAD) operating at 295 or 210 nm. Bare fused-silica capillaries with 50 and 150 µm I.D. were purchased from Agilent. The capillary temperature was set at 25 °C for all the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used.

2.3. Sweeping

For the sweeping-MEKC procedure, the capillary (60 cm, 50 µm I.D.) was initially conditioned with BGE (20 mM NaH_2PO_4, 150 mM SDS with 15 % ACN at pH 2.28 adjusted with diluted phosphoric acid). All the analytes were dissolved in a 0.3 mM NaOH solution (pH 10.5) and injected hydrodynamically at 50 mbar for 100 s. After sample injection, a voltage of -17.5 kV was applied for both sample preconcentration and subsequent separation.

2.4. LVSS-CZE

In the LVSS-CZE procedure, the capillary (53 cm, 50 µm I.D.) was initially conditioned with BGE (30 mM $Na_2B_4O_7$ at pH 9.5 adjusted with NaOH 0.1 M). All the analytes were in a 0.3 mM NaOH solution (pH 10.5) and injected hydrodynamically at 50 mbar for 500 s. After sample injection, a negative voltage of -10 kV was applied, and the sample stacking began. When 95 % of the current was reached, the voltage was stopped and a positive voltage of 20 kV was then applied to separate the compounds.

2.5. FASI-CZE

In the FASI-CZE procedure, the capillary (53 cm, 50 µm I.D.) was initially conditioned with BGE (30 mM $Na_2B_4O_7$ at pH 9.5 adjusted with NaOH 0.1M). A plug of MeOH:ACN (50:50 v/v) was then introduced at 50 mbar for 120 s before sample injection. A voltage of -5 kV was then applied to electrokinetically inject the analytes which were dissolved in a 0.3 mM NaOH solution (pH 10.5). When 95 % of the current was reached, the voltage was stopped and a positive voltage of 20 kV was then applied to separate the compounds.

2.6. In-line SPE-CZE

2.6.1. Construction of the analyte concentrators

The analyte concentrator (AC) which contained the SPE sorbent was made in-house. For its preparation, a method described in a previous work [37] was followed. A 67 cm (50 µm I.D. × 360 µm O.D.) fused-silica capillary was activated and then cut into two pieces of 7.5 cm (inlet capillary) and 59.5 cm

(separation capillary) in order to insert the AC between them. The AC used had a length of 2 mm (150 µm I.D. × 360 µm O.D.) and it was filled with Oasis HLB sorbent (60 µm average particles size). The entire process of fabricating the concentrator was monitored under a microscope. Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow restriction before the analysis.

2.6.2. In-line SPE-CZE methodology

The SPE-CE-UV capillary was conditioned with BGE (30 mM $Na_2B_4O_7$ at pH 9.5 adjusted with NaOH 0.1M) for 15 min at the beginning of each day. Before each injection, the capillary was first conditioned by consecutive flushes of ACN for 2 min, and Milli-Q water at pH 3 adjusted with diluted phosphoric acid for 3 min at 930 mbar. The sample was then loaded at pH 3 adjusted with diluted phosphoric acid at 930 mbar for 60 min (ca. 77.5 µL using Hagen-Poiseuille equation). The next step was a sample clean-up with BGE solution by applying 930 mbar for 3 min, in order to eliminate non-retained molecules and equilibrate the capillary before elution and the electrophoretic separation. Afterwards, the retained analytes were eluted by injecting a plug of ACN, using a pressure of 50 mbar for 30 s (34 nL) followed by an injection of BGE at 50 mbar for 150 s to push the elution plug through the concentrator to the beginning of the separation capillary (ca. 9 cm using the Hagen-Poiseuille equation). Finally, a voltage of 15 kV was applied to perform the electrophoretic separation. After each injection, the capillary was rinsed for 2 min with ACN and 2 min with Milli-Q water, in order to avoid carry-over problems between consecutive injections.

3. RESULTS AND DISCUSSION

In this work, four different preconcentration strategies in CZE and MEKC to improve the sensitivity for the determination of parabens were evaluated. According the basic requirements of each preconcentration technique, the conditions were selected to separate the compounds in CZE and MEKC. In the literature, different BGE solutions have been used for the separation of parabens by CE. For the CZE separation, sodium borate at basic conditions (pH 8-10) is the most commonly used BGE [11, 12, 19], whereas for MEKC separation, the BGEs normally used are sodium borate and sodium phosphate, either at acidic or basic conditions containing SDS [13-15, 18, 24].

3.1. Sweeping-MEKC

Sweeping is based on the analytes' ability to partition into a PS in MEKC. High concentration efficiency in sweeping can be obtained for neutral and weak basic analytes using an anionic PS and suppressed EOF in a low pH BGE provided by a phosphate buffer (pH 2–3) [32]. Therefore, sodium dihydrogenphosphate containing SDS was selected as the acidic micellar BGE to evaluate the separation of six model compounds: MP, EP, PP, IPP, BP, and BzP. The variation of sodium dihydrogenphosphate concentration (20-50 mM) at pH 2.28 adjusted with diluted phosphoric acid, and micellar concentration (70-100 mM SDS) in the BGE were examined in order to find out the optimal conditions for the separation. The best separation was obtained using 20 mM of sodium dihydrogenphosphate, 70 mM of SDS at pH 2.28, and the optimal voltage was found to be -17.5 kV, with an analysis time of 10 min.

As reported by Quirino *et al*. [39], the sweeping preconcentration can be performed in a homogeneous or in a heterogeneous electric field. In other words, the sample can be prepared with an equal or different conductivity to the BGE conductivity.

Sweeping in a homogeneous electric field was first investigated. The conductivity of the standard sample containing the parabens was adjusted to match the conductivity of the BGE by the addition of sodium dihydrogenphosphate at pH 2.28 adjusted with diluted phosphoric acid. The sample was hydrodynamically injected into the capillary at 50 mbar for 3 different times, 15, 50 and 100 s, and then the separation voltage of -17.5 kV was applied.

As is well known, analytes with a large interaction with the micelles will be swept better than those with a low interaction [32]. At an injection time of 15 s, BzP, BP, IPP, and PP were focused at a certain grade. However, EP and MP migrated as small and broad peaks, because they do not have sufficient interaction with the micelles for proper accumulation to occur. The increase of the injection time to 50 s involved a focusing effect only for BP and BzP, while the other compounds were not focused and a loss of resolution was also observed. At an injection time of 100 s, only BP was focused.

To improve sweeping ability, the effect of varying the SDS concentration in the buffer was examined over the 70-150 mM concentration range (sample injection at 50 mbar for 50 s, 1000 ng/mL) [40-43]. As can be seen in Figure 1A, higher SDS concentration improved the sweeping efficiency because of the higher interaction of the analytes with the micelles, resulting in a larger retention factor that shortened the swept

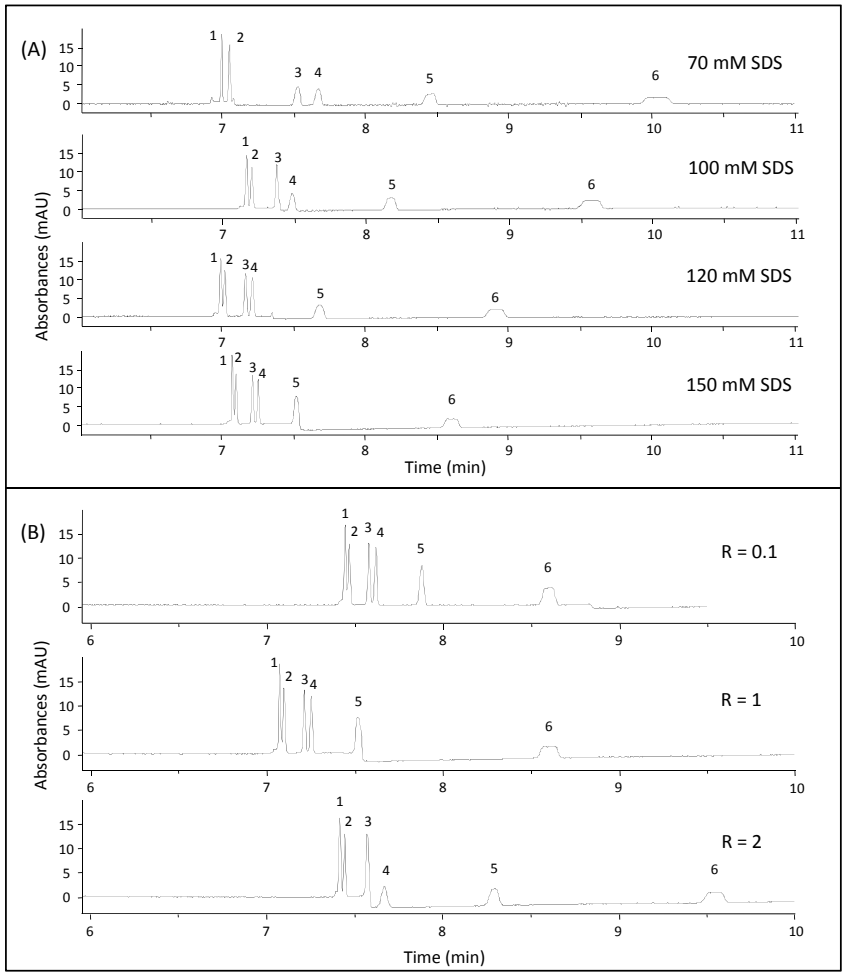

Figure 1. Optimization of the sweeping preconcentration technique. (A) Effect of varying SDS concentration in the BGE. Conditions: BGE, 20 mM NaH_2PO_4 (pH 2.28) with different SDS concentrations: 70 mM, 100 mM, 120 mM, and 150 mM; sample containing the analytes at a concentration of 1000 ng/mL; sample injection at 50 mbar for 50 s; separation voltage, -17 kV. (B) Effect of the sample matrix conductivity. Conditions: BGE, 150 mM SDS in 20 mM NaH_2PO_4 (pH 2.28); sample containing the analytes at a concentration of 1000 ng/mL; sample injection at 50 mbar for 50 s. Conductivity ratio of the sample and the BGE: R=0.1, R=1, and R=2. Peak designation. (1) BzP, (2) Bp, (3) IPP, (4) PP, (5) EP, and (6) MP.

zone. When the SDS concentration was 150 mM, BzP, BP, IPP, and PP were totally swept, whereas EP had a more moderate focusing effect, and MP was not focused.

As demonstrated by Quirino and Terabe [39], the focusing effect for less hydrophobic compounds is better in a heterogeneous field due to the combination of sweeping and stacking

mechanisms. Taking that study into account, the influence of changing the sample conductivity on the sweeping was investigated. Therefore, the conductivity ratio between the sample and the BGE (R= sample conductivity/ BGE conductivity) was modified. To carry out this study, the analytes were dissolved in Milli-Q water adjusted at pH 2.28 with diluted phosphoric acid, leading to a conductivity ratio (R) in that case of 0.1, and in sodium dihydrogenphosphate at pH 2.28 adjusted with diluted phosphoric acid to match (R=1) and double (R=2) the conductivity of the BGE. As can be observed in Figure 1B, a better focusing effect was obtained by injecting a sample with a lower conductivity than the BGE (R= 0.1), although a striking peak broadening was still apparent for MP.

In the reported literature, it has been demonstrated that the sample and the BGE pH values play an important role in the sweeping preconcentration [32]. The sample pH involves a different ionization degree of the compounds which produce a variation in the focusing effect. Since the pKa values of this group of compounds ranged from 8.0 to 9.5, the influence of different sample pH values was examined. This was evaluated by preparing the analytes in Milli-Q water at different pH values: 2.28 (to match the BGE), 5.50, and 10.50. The best results for all the six compounds relating to the highest peak height and peak efficiency were obtained at the basic pH value tested (10.5). In the optimum conditions, sample prepared in Milli-Q water at pH 10.5, the compounds were completely in their anionic form (pKa 8-9.5) and the sample had a lower conductivity than the BGE ($R \approx 0.1$). In this case, the preconcentration is due to a triple mechanism, the combination of LVSS, dynamic pH junction and sweeping (LVSS-DypH-sweeping). To optimize the pH of the BGE, different values were tested (2.00, 2.28, 2.50, and 3.00). From the obtained results, it could be observed that the increase of the pH involved a decrease of the focusing effect, as well as a loss of resolution. At pH 3, both BP and BzP, as PP and IPP were not separated. The best results were obtained when the pH had a value of 2.28.

It should be pointed out that the optimal BGE composition for sweeping negatively affected the resolution. In order to improve its resolution, the addition of different organic modifiers to the BGE, such as MeOH and ACN, was evaluated. A significant improvement in resolution was obtained with the presence of ACN, and percentages of 10%, 15% and 20 % (v/v) were tested. An optimal value of 15 % of ACN was selected as it provided the best results in terms of resolution, analysis time, and sweeping focusing.

The influence of the sample injection time on the sweeping efficiency was studied in the range of 15 to 250 s at 50 mbar. The peak height for the different compounds increased when extending the injection time only up to 100 s. When longer injection times were used, a loss of efficiency and resolution occurred, involving a decrease in the peak height. Taking this into account, an injection time of 100 s was selected which provided a proper resolution and peak efficiency with the highest stacking sensitivity.

Under the optimal conditions obtained for sweeping, the sensitivity was enhanced (SEF) by 17- to 29-fold, and 18- to 28-fold in terms of LODs and peak area, respectively. The SEF_{LODs} values were calculated as a ratio of the LOD values obtained by the normal hydrodynamic injection (corresponding to loading the sample into the capillary at 50 mbar for 5 s) and the ones obtained for the preconcentration techniques. The SEF_{area} values were calculated simply by getting the ratio between the peak areas obtained when a preconcentration technique was used, and those obtained for the normal hydrodynamic injection, and then the quotient is multiplied by the dilution factor. Figure 2A shows an electropherogram obtained for a standard sample containing the analytes at a concentration of 100 ng/mL with the sweeping-MEKC procedure.

Even though the term sweeping was used for the developed preconcentration strategy, the term used in the literature is different when sweeping is used in combination with other preconcentration techniques. For instance, when a sample matrix with low conductivity is used, the technique has been referred to as LVSS-sweeping [44, 45] or dynamic pH junction-sweeping, when the sample matrix has a different pH from the BGE [46, 47]. Cheng *et al*. [40] used a low conductivity sample matrix with a different pH of the BGE for the determination of dipeptides and, in that case, the reported name was LVSS-DypH-sweeping. That system involved a combination of LVSS, dynamic pH junction and sweeping preconcentration, as is the case in the present work.

3.2. LVSS-CZE

To evaluate separation by CZE, four parabens were chosen as model compounds: MP, EP, PP and BP. A borate buffer at basic pH was selected as BGE. The variation of the sodium borate concentration (20-50 mM sodium borate) and pH (from 9.0 to 10.5) were examined to find out the optimal conditions for the separation of the compounds. The best separation was obtained with the use of 30 mM of sodium borate at pH 9.5, and the optimal separation voltage was found

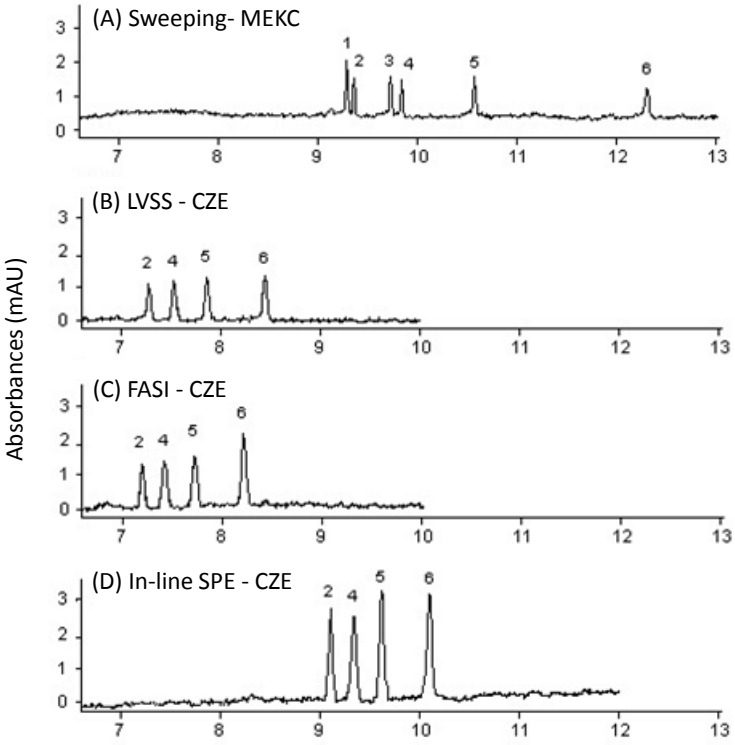

Figure 2. Electropherogram of a standard solution of the parabens in the four preconcentration approaches under their optimal conditions. (A) Sweeping, sample containing the analytes at a concentration of 100 ng/mL. (B) LVSS, sample containing the analytes at a concentration of 20 ng/mL. (C) FASI, sample containing the analytes at a concentration of 10 ng/mL. (D) In-line SPE-CE sample containing the analytes at a concentration of 0.2 ng/mL. Peak designation. (1) BzP, (2) Bp, (3) IPP, (4) PP, (5) EP, and (6) MP. The experimental conditions are reported in the text.

to be 20 kV, with an analysis time of 9 minutes.

For LVSS, parabens were dissolved in a 0.3 mM NaOH solution (pH 10.5), since a low conductivity sample matrix is necessary in order to have the proper sample stacking conditions, and the compounds should be in their anionic form in order to be separated by CZE [27]. Under these conditions, parabens stacked after passing through the boundary between the sample zone and the BGE, while the sample matrix was electroosmotically pushed out of the capillary after the application of the anodic stacking voltage (cathode in the inlet). When 95 % of the original current was reached, the anodic voltage was stopped and the polarity was switched. Parabens migrated to the cathode because, at this pH, the cathodic EOF value was higher than the

electrophoretic velocity of the compounds towards the anode. The main parameters to be optimized in LVSS were the sample injection time and the anodic stacking voltage.

A standard solution containing the four parabens (BP, PP, EP, MP) at a concentration of 500 ng/mL in a 0.3 mM NaOH solution (pH 10.5) was hydrodynamically injected at 50 mbar in order to fill 25, 50, 75 and 100 % of the total capillary volume, corresponding to hydrodynamic injection times of 132, 252, 372 and 500 s, respectively. For this study, the anodic stacking voltage was kept at -5 kV and, for CE separation, a voltage of 20 kV was applied. Figure 3A shows the increment of the SEF_{area} with the increase of the injection time for each compound. An injection time of 500 s was selected because it allowed the whole capillary to be filled with sample obtaining the higher focusing effect, and no band-broadening was observed.

The magnitude of the anodic stacking voltage was studied in the range of -2 kV to -25 kV, while the sample matrix was pumped out of the capillary until the current reached the 95 % of the initial value (when the capillary is totally filled with BGE). As can be seen in Figure 3B, the optimum was found to be -10 kV because the maximum concentration factor in terms of peak area (SEF_{area}) was obtained in a relatively short time (around 3.5 min).

When higher stacking voltages were tested, improvements of the concentration factors were not observed and also the method was less precise, since the monitoring of the current was more difficult because it slowed down dramatically. However, lower stacking voltages increase the stacking time, which can produce a loss of the analytes during removal of the sample matrix [29].

Under the optimal conditions obtained for LVSS, the sensitivity was enhanced by 53- to 77-fold and 92- to 109-fold in terms of LODs and peak area, respectively. Figure 2B shows the electropherogram obtained for a standard sample containing the analytes at a concentration of 20 ng/mL with the LVSS-CZE procedure.

3.3. FASI-CZE

For FASI, parabens were also prepared in a 0.3 mM NaOH solution (pH 10.5) to ensure the proper sample stacking conditions [27]. In this case, we also hydrodynamically injected a plug of low conductivity solvent prior to the sample injection. In this way, the electrical field established across the solvent plug was several hundred times higher than that in the BGE favouring charged analytes being injected at high velocity [30]. A negative voltage was then applied to inject the parabens into the solvent plug. Parabens stacked at

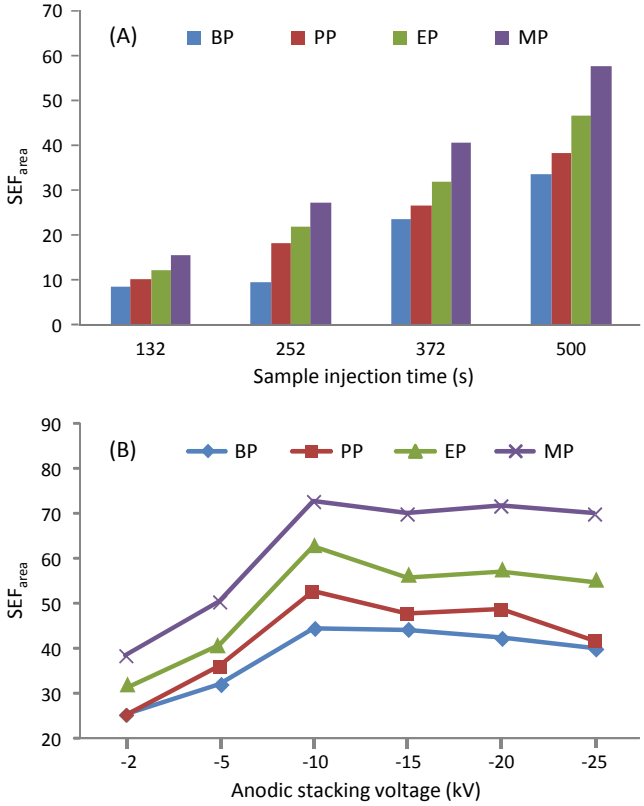

Figure 3. Optimization of the LVSS preconcentration technique. (A) Effect of the hydrodynamic injection time on the SEFarea. Sample containing the analytes at a concentration of 500 ng/mL; (B) Effect of the anodic stacking voltage applied on the SEFarea. Sample containing the analytes at a concentration of 100 ng/mL. Conditions: BGE, 30 mM sodium borate (pH 9.5), separation voltage, 20 kV.

the boundary between the solvent plug and the BGE while the solvent plug was electroosmotically pushed out of the capillary. When 95 % of the original current was reached, the anodic voltage was stopped and the polarity was switched. Parabens migrated to the cathode because at this pH the cathodic EOF value was higher than the electrophoretic velocity of parabens towards the anode. The main parameters to be optimized in FASI were the solvent plug composition and its hydrodynamic injection time, and the injection voltage of the sample.

The solvent plug provides high electric field strength at the beginning of the capillary, which facilitates the injection of the analytes, as stated previously. The most widely used solvents as a

solvent plug are water and mixtures of water with methanol or acetonitrile. In this study, the solvents tested were: H_2O, H_2O at pH 10.5; MeOH; MeOH:H_2O at pH 10.5 (50:50 v/v); ACN:H_2O at pH 10.5 (50:50 v/v); MeOH:ACN (50:50 v/v); and ACN. They were selected for their conductivities which are lower than the conductivity of the BGE. Experimentally, each solvent plug was hydrodynamically injected at 50 mbar for 50 s, then the sample prepared in a 0.3 mM NaOH solution containing the parabens at a concentration of 500 ng/mL was electrokinetically injected at -5 kV until the current intensity was 95 % of its original value. The voltage was then stopped and a positive voltage of 20 kV was applied to separate the compounds. As can be seen in Figure 4A, the best results in terms of peak height were obtained when a plug of MeOH:ACN (50:50 v/v) was introduced into the capillary before the sample injection. Therefore, this mixture was used as the solvent plug for further experiments. For convenience, this MeOH:ACN (50:50 v/v) plug is referred to as the solvent plug in the discussion below.

To study the effect of the length of the solvent plug in the sensitivity enhancement several injection times between 50 and 400 s at 50 mbar were tested, and the sample with the parabens at a concentration of 200 ng/mL prepared in a 0.3 mM NaOH solution was injected at -5 kV until the intensity reached 95% of the original current. Figure 4B shows an increase of the peak height versus the injection time of the solvent up to 400 s. However, from 200 s onwards the increase was not significant. Therefore, as a compromise between preconcentration, efficiency and analysis time, 200 s was selected as the optimal injection time for the solvent plug.

The influence of electrokinetic sample injection voltage on sensitivity was also investigated. The sample injection voltage was increased from -5 kV to -20 kV. Injection voltages above -5 kV caused a peak broadening and loss of efficiency and, in addition, involved more difficulty in monitoring of the current, and therefore more ireproducibility between injections. Taking these results into account, -5 kV was selected as sample injection time by FASI.

Under the optimal conditions obtained for FASI, the sensitivity was enhanced by 105- to 120-fold, and 186- to 232-fold in terms of LODs and peak area, respectively. Figure 2C shows the electropherogram obtained for a standard sample containing the analytes at a concentration of 10 ng/mL by using the FASI-CZE procedure.

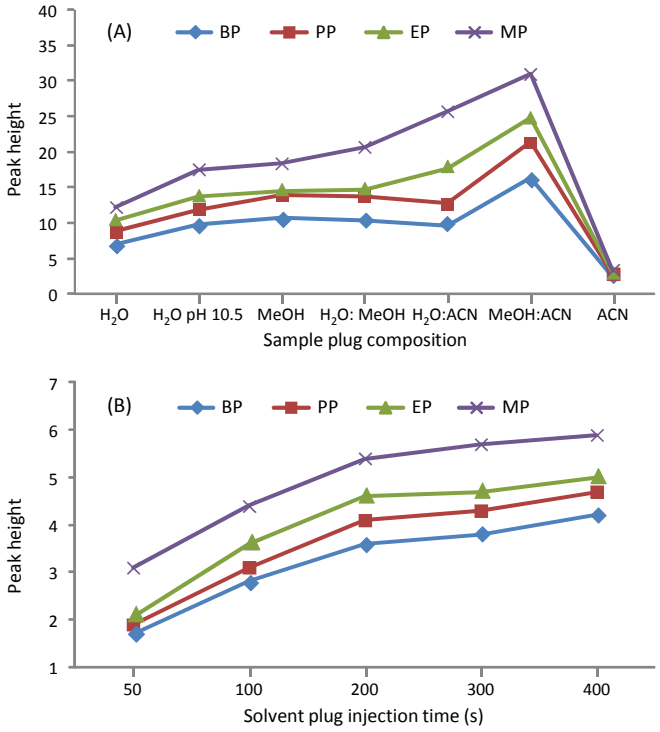

Figure 4. Optimization of the FASI preconcentration technique. (A) Effect of the solvent plug composition on the peak height. Sample containing the analytes at a concentration of 500 ng/mL. (B) Effect of the hydrodynamic injection time of the solvent plug on the peak height. Sample containing the analytes at a concentration of 200 ng/mL. Conditions: BGE, 30 mM sodium borate (pH 9.5), separation voltage, 20 kV. Other experimental conditions are reported in the text.

3.4. In-line SPE-CZE

Chromatographic preconcentration techniques, such as in-line SPE-CZE, allow high sensitivity enhancement factors to be achieved, due the large volume of sample that can be loaded into the capillary [28]. In this study, Oasis HLB sorbent was used as the extraction sorbent, since different authors have previously used this sorbent in off-line SPE for the determination of parabens in environmental water samples by HPLC or CE [9,22]. In order to achieve the optimal performance for the in-line SPE-CZE method, several parameters were investigated, including the sample pH, the elution solvent and its volume, and the sample loading time.

The sample pH involved a different ionization degree of the compounds, and it played an important role in the retention of the analytes into the

sorbent. Since the pKa values of this group of compounds ranged from 8.0 to 9.5, the influence of having a different pH value for the sample (pH 3.0, 6.0, and 10.5) was examined. For this study, standard solutions of mixtures containing 50 ng/mL of each paraben were injected for 5 min at 930 mbar and then eluted by injecting a plug of MeOH for 20 s at 50 mbar [9,22]. The results are shown in Figure 5A, where the response for the different compounds, in terms of peak area, is represented at the different sample pH values tested. As can be observed, the higher retention in the Oasis HLB sorbent was obtained at acidic pH value of pH 3.0. At this pH, compounds are in their non-anionic form, and they are retained by the strength of their hydrophobic interactions with the sorbent face.

To study the elution step, different organic solvents reported in the literature were tested, all at 50 mbar for 20 s: MeOH, ACN, acetone, and MeOH with 5 % NH_4OH [6,9,22,37]. As can be seen in Figure 5B, the best results in terms of peak area were obtained when ACN was used. The elution volume was then studied by introducing ACN with a hydrodynamic injection from 20 to 60 s (23 to 69 nL) at 50 mbar. It was observed that the peak area for the different compounds increased with the elution volume up to 30 s (34 nl). When higher plugs of ACN were used, a loss of peak efficiency and resolution were obtainned, which involved a peak broadening and a decrease of the peak height. Taking this into account, an elution volume of 34 nL (30 s) was selected.

The introduction of a large sample volume is the most important factor to obtain low LODs. In order to load the maximum sample volume and establish the maximum capacity of the Oasis HLB sorbent, the sample loading time at 930 mbar was investigated using a solution of 1 ng/mL. Figure 5C shows the peak area of the eluted compounds as a function of the sample loading time, which was increased from 5 to 60 min (i.e. 6.5-77.5 µl) at 930 mbar. A linear relation of the peak area versus sample loading time was observed up to 60 min, indicating that the breakthrough volume was not reached. Although this trend of increasing the response with higher injection volumes of the sample was observed, an injection time of 60 min was selected as the optimal value. In this selection, the fact that the introduction of a higher sample volume would prolong the analysis time too much was also considered.

Under the optimal conditions obtained for in-line SPE-CE, the sensitivity was enhanced by 12600- to 18400-fold, and 17700- to 19000-fold in terms of LODs and peak area, respectively. Figure 2D shows the electropherogram obtained

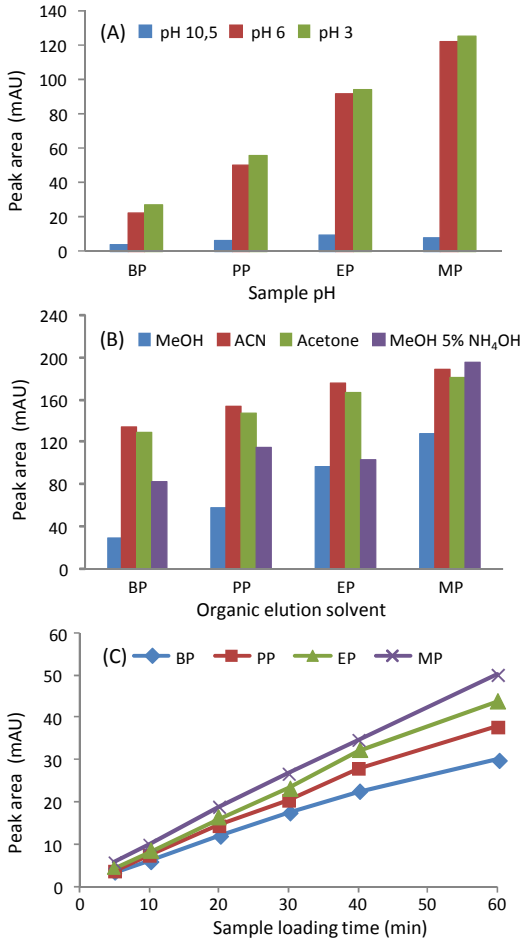

Figure 5. Optimization of the in-line SPE-CZE preconcentration technique. (A) Effect of the sample pH on the peak area. Sample containing the analytes at a concentration of 50 ng/mL. (B) Effect of the organic elution solvent on the peak area. Sample containing the analytes at a concentration of 50 ng/mL. (C) Study of the sample loading time. Sample containing the analytes at a concentration of 1 ng/mL. Conditions: BGE, 30 mM sodium borate (pH 9.5), separation voltage, 15 kV. Other experimental conditions are reported in the text.

with the in-line SPE-CZE procedure for a standard sample containing the analytes at a concentration of 0.2 ng/mL.

3.5. Validation of the four preconcentration techniques

Standard solutions were analysed by each technique in order to determine

the linearity of each compound, the calibration graphs, repeatability, reproducibility, the sensitivity enhancement factors and the LODs. The results are shown for each preconcentration technique in Table 1.

The linear responses were good in the 50-500 ng/mL, 15-500 ng/mL, 10-500 ng/mL, and 0.05-5.0 ng/mL concentration range for sweeping, LVSS, FASI, and in-line SPE-CZE, respectively. The calibration curves were calculated by using five points at different concentrations and each standard was injected three times. The correlation coefficients (R^2) were higher than 0.997 for all compounds for the four techniques. The day-to-day reproducibility and the repeatability of the four preconcentration techniques, expressed as relative standard deviations (RSDs), was calculated by performing three and ten replicate determinations on five different days, respectively. The reproducibility and repeatability study was carried out with a standard solution at a concentration of 200 ng/mL for sweeping and LVSS, 100 ng/mL for FASI, and 1 ng/mL for in-line SPE-CZE.

As can be observed in Table 1, the RSD values of the migration time and the peak area in terms of reproducibility and repeatability for sweeping, LVSS, and FASI were higher than those obtained for in-line SPE-CZE. This could be explained by the fact that in the electrophoretic preconcentration techniques, the voltage applied plays an important role in both the preconcentration and the separation stage of the analytes. However, in the in-line SPE-CZE, as the injection is not performed electrokinetically and the preconcentration effect does not depend on the current control, the technique is more precise.

In order to compare the effectiveness of the preconcentration techniques studied, the enhancement of sensitivity for the four techniques was compared. The sensitivity enhancement factors were evaluated in terms of peak area and also by the LODs obtained. For sweeping, the average enhancement factors were found to be 24-fold and 22-fold in LODs and peak area, respectively. These results are in agreement with the characteristics of the analytes studied. Parabens had a moderate interaction with the SDS and they were only preconcentrated to a significant extent when a small sample plug (100 s of hydrodynamic injection) was injected with larger sample plugs leading to loss of resolution. For LVSS-CZE, the average enhancement factors were found to be 61-fold, and 99-fold in LODs and peak area, respectively. It is important to note that this is the maximum improvement in sensitivity that could be obtained in LVSS because this was achieved by filling the capillary volume completely. For the FASI-CZE preconcentration technique, the ave-

Table 1. Linearity, calibration curves, LODs, repeatability and reproducibility (RSD%) for migration time and peak area, and sensitivity enhancement factors (SEF) for standard samples obtained for the different developed methodologies.

Compounds	Linearity (ng/mL)	Calibration curve	R^2	LOD (ng/mL)	Repeatability (% RSD) (n=10)		Reproducibility (% RSD) (n=5)		[a]SEF_{LOD}	[b]SEF_{area}
					Migration time	Peak area	Migration time	Peak area		
Sweeping					200 ng/mL	200 ng/mL	200 ng/mL	200 ng/mL		
BzP	50-500	y = 16.53x - 0.09	0.998	18	1.0	5.6	2.6	10.1	29	23
BP	70-500	y = 36.14x - 0.88	0.999	22	0.7	4.1	2.6	11.1	25	20
IPP	70-500	y = 22.39x - 0.11	0.998	20	0.8	5.4	2.4	12.8	25	28
PP	70-500	y = 18.63x - 0.03	0.999	22	0.9	4.4	2.4	16.9	25	18
EP	70-500	y = 18.70x + 0.42	0.998	20	0.9	6.8	2.2	13.7	20	21
MP	90-500	y = 22.20x + 0.37	0.999	27	0.9	5.5	2.0	14.6	17	19
LVVS					200 ng/mL	200 ng/mL	200 ng/mL	200 ng/mL		
BP	15-500	y = 177.30x + 0.05	0.999	4	1.8	7.3	2.2	13.2	60	92
PP	15-500	y = 199.45x - 0.22	0.998	4	3.3	6.6	1.9	16.1	53	92
EP	15-500	y = 238.53x + 0.28	0.999	3	2.0	5.2	2.9	18.6	77	100
MP	15-500	y = 295.00x - 1.01	0.999	4	2.2	7.3	2.5	15.5	53	109
FASI					100 ng/mL	100 ng/mL	100 ng/mL	100 ng/mL		
BP	10-500	y = 0.73x - 4.77	0.997	2	1.7	5.9	2.2	15.2	120	186
PP	10-500	y = 1.02x - 11.09	0.998	2	2.1	6.6	2.7	16.8	105	195
EP	10-500	y = 1.17x - 11.65	0.998	2	1.8	7.8	2.3	17.4	115	211
MP	10-500	y = 1.55x - 21.47	0.998	2	1.2	7.0	2.0	17.4	105	232
In-line SPE					1 ng/mL	1 ng/mL	1 ng/mL	1 ng/mL		
BP	0.1-5.0	y = 32.37x - 2.27	0.999	0.02	0.5	2.0	1.4	5.1	14400	17700
PP	0.1-5.0	y = 37.09x - 4.11	0.999	0.02	0.5	3.0	1.1	4.9	12600	19000
EP	0.05-5.0	y = 40.59x - 4.82	0.999	0.01	0.6	2.4	1.8	5.2	18400	19000
MP	0.05-5.0	y = 42.09x + 6.82	0.999	0.01	0.6	3.2	1.7	5.4	16100	18000

[a] SEF_{LOD} (Sensitivity enhancement factor for LOD) = ($LOD_{preconcentration\ technique}$/$LOD_{normal\ hydrodynamic\ injection}$).

[b] SEF_{area} (Sensitivity enhancement factor for peak area) = dilution factor × ($area_{preconcentration\ technique}$/$area_{normal\ hydrodynamic\ injection}$).

rage enhancement factors were found to be 112-fold, and 206-fold in LODs and peak area. As was expected, the SEFs obtained in FASI were higher than those of LVSS, since the analytes were electrokinetically injected.

The limits of detection (LODs) achieved were calculated at signal/noise ratio of 3. Regarding the SEF obtained, higher LODs were obtained for sweeping from 18-27 ng/mL, followed by LVSS which achieved LODs from 3-4 ng/mL. Both these techniques were performed by a hydrodynamic injection of the sample. FASI achieved LODs of 2 ng/mL, due to the electrokinetically injection of the analytes. For the chromatographic preconcentration technique studied, the in-line SPE-CZE system, the SEFs obtained were 1000 or 100 times higher than those of stacking techniques, and so the LODs were much lower. These electrophoretic and chromatographic techniques studied are not comparable in terms of SEF and LODs, since the amount of sample injected in both strategies is very different. The chromatographic technique studied allows the injection of 60 min of sample, a volume of 77.5 µL, and the maximum volume of sample injected by LVSS is 0.6 µL, which explains the differences between the LODs and SEF achieved. For the in-line-SPE-CZE, the average enhancement factors were found to be 15400-fold, and 18500-fold in LODs and peak area, respectively, and the LODs obtained ranged from 0.01 to 0.02 ng/mL. However, also it should be taken into account the time needed for each preconcentration technique. The total analysis time when an electrophorethic preconcentration technique is applied does not exceed the 30 min, however the chromatographic preconcentration technique is carried out with a total analysis time of approximately 80 min. This long time is mainly attributed to the sample loading time which is 60 min.

As mentioned previously, parabens have already been preconcentrated and separated by electrophoretic preconcentration techniques. Blanco *et al.* [21,22], after an off-line SPE procedure, applied a LVSS combined with NACE for the quantification of parabens in water samples. The authors achieved an improvement of LODs of 10- to 25-fold depending on the analytes for the LVSS-NACE-DAD preconcentration for standard samples. Although these improvements are lower than those obtained with the electrophoretic preconcentration strategies used in the present study, they achieved lower LODs, around 0.8 and 2.1 ng/ml. This can be explained by the use of a capillary of 75 µm of I.D, which allowed the introduction of a higher sample volume (2.4 µL instead of 0.7 µL that was the volume introduced in our case). In another study performed by

He *et al.* [24], the combination of LVSS with MEKC to determine parabens in some cosmetic products was carried out. The LODs obtained with this technique were around 50 ng/mL, which are higher than those obtained with the elec-trophoretic techniques studied in the present study.

The different electrophoretic preconcentration methods (sweeping-MEKC, LVSS-CZE, and FASI-MEKC) used for analysing this group of parabens provided adequate linearity, repeatability and reproducibility and a different range of focusing effect. These methodologies could be suitable for the determination of parabens in different kinds of samples, such as pharmaceuticals, cosmetics, food and biological samples, depending on the concentration expected and also on the sample matrix.

Concerning the in-line SPE-CZE method developed, the great potential of the method comes from the high sensitivity enrichment factors obtained (with average values of 15400-fold and 18500-fold, in LODs and peak area, respectively). Thus, this method is among the most sensitive for the analysis of parabens by CE. LODs of the optimized in-line SPE-CZE method, using conventional UV-detection, achieved instrumental sensitivity similar to GC-MS or GC-MS/MS [7, 8] and HPLC-MS/MS [6,9]. However, the LODs achieved are not low enough to reach the levels at which these compounds are usually found in environmental waters, so a pretreatment procedure is usually needed. The in-line SPE-CZE method developed can be considered as an alternative to the chromategraphic techniques for the analysis of environmental water samples. However, in this case, a previous off-line preconcentration step, such as solid-phase extraction or liquid-liquid extraction, would be necessary.

4. CONCLUSIONS

This paper discusses and compares four techniques to preconcentrate a group of parabens. The techniques evaluated are three electrophoretic preconcentration techniques: two of them based on stacking applied to the CZE method, LVSS and FASI, and the other based on sweeping; and one chromatographic preconcentration technique, namely in-line SPE-CE. For the electrophoretic preconcentration techniques, the LODs obtained were in the range of 18–27 ng/mL, 3-4 ng/mL, and 2 ng/mL for sweeping, LVSS and FASI, respectively. The in-line SPE-CE method, under optimized conditions, achieved sensitivity enrichment factors for these compounds in standard samples up to 18400-fold for LODs and 19000-fold for peak area, and LODs ranging between 0.01 and 0.02 ng/mL were obtained. The proposed procedures are simple,

selective, repeatable and effective, as demonstrated by the sensitivity and precision studies carried out. This study could provide a good starting point for the determination of parabens in different types of samples, such as pharmaceutical, cosmetics, food, biological or environmental samples, depending on the concentration expected and the complexity of the sample.

Acknowledgements

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3.3.2. Discussion of results

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The results presented in this final section demonstrate that the use of CE with a preconcentration technique, either electrophoretic or chromatographic, is a reliable alternative to more conventional analytical methods for determining parabens.

To obtain these results, the study focused on the comparison of different preconcentration techniques, sweeping, LVSS, FASI and in-line SPE-CE. With a sweeping preconcentration technique, the compounds had to be separated by MEKC under acidic conditions in order to obtain high efficiency for weak basic analytes using an anionic PS and suppressed EOF. A BGE containing sodium dihydrogen phosphate and SDS at pH 2.28 was used to optimize the separation, and the best conditions were obtained by using 20 mM of sodium dihydrogen phosphate and 70 mM of SDS. For the application of LVSS, FASI and in-line SPE, parabens had to be in their anionic form in order to be separated by CZE (pKa of parabens 8.0-9.5). A basic BGE was then used, in particular, a BGE containing sodium borate at high pH (from 9.0 to 10.5), in order to optimize the separation. The best conditions were found to be with the use of 30 mM of sodium borate at pH 9.5.

The optimized parameters for the each preconcentration technique studied in this section are shown in Table 3.3.1.

Table 3.3.1. Parameters optimized for sweeping, LVSS, FASI and in-line SPE-CE.

Sweeping	**FASI**
• SDS concentration in the BGE • Composition and pH of the sample • pH of the BGE • Organic solvent in the BGE • Sample hydrodynamic injection	• Solvent plug composition • Hydrodynamic injection of solvent plug • Sample injection voltage
LVSS	**In-line SPE-CE**
• Sample hydrodynamic injection • Anodic stacking voltage	• Sample pH • Composition of the elution solvent • Volume of the elution solvent • Sample loading time

In sweeping, the SDS concentration was optimized because the compounds studied were far from completely swept with the optimized BGE for the MEKC separation. An increase of the SDS concentration in the BGE could therefore improve the sweeping ability. The concentration of SDS in the BGE was increased up to 150 mM and this high SDS concentration improved the sweeping efficiency because of the higher interaction

of the analytes with the micelles, resulting in a high retention factor that shortened the swept zone. This high SDS concentration caused a loss of resolution and in order to improve it, the addition of an organic solvent to the BGE was studied. The best results in terms of resolution, efficiency, focusing effect and analysis time were obtained when 15 % of ACN was added to the BGE. The common protocol for sweeping neutral and ionic analytes is based on preparing the sample in a matrix with a similar conductivity to the BGE and also at the same pH. However, under these conditions, not all the compounds were focused so an exhaustive study of the sample conductivity and sample pH was performed. The best focusing effect was obtained when the sample was prepared in a 0.3 mM NaOH solution (pH 10.5). Under these sample conditions, conductivity of the sample was lower than the conductivity of the BGE and had a different pH to the BGE. Under the above optimized conditions, the preconcentration is due to the triple mechanism of the combination of LVSS, dynamic pH junction and sweeping (LVSS-DypH-sweeping).

For LVSS and FASI, parabens were dissolved in a 0.3 mM NaOH solution (pH 10.5), as a low conductivity sample matrix is a requirement of these preconcentration techniques. For LVSS, the main parameter to be optimized was the hydrodynamic sample injection time and the highest focusing effect was obtained when the capillary was completely filled with the sample (500 s at 50 mbar). An anodic stacking voltage, evaluated in the range between -2 kV and -25 kV, was applied to remove the sample matrix out of the capillary, and the polarity was switched when the current reached 95 % of the initial value (when the capillary is totally filled with BGE). The maximum concentration factor in terms of peak area (SEF_{area}) was obtained when the applied anodic stacking voltage was -10 kV. Higher stacking voltages did not cause an improvement of the concentration factor and, moreover, the monitoring of the current became more difficult because it slowed down dramatically. In contrast, lower stacking voltages increased the stacking time, which produced a loss of the analytes during the removal of the sample matrix, so lower concentration factors were achieved.

In the case of FASI, there were different parameters which affected the pre-concentration ability, namely the solvent plug composition, its hydrodynamic injection and the sample injection voltage. The low conductivity solvent plug provided high electric field strength at the beginning of the capillary, which facilitates the injection of the analytes [1]. Different mixtures of water with methanol or acetonitrile were tested as solvent plugs and the best results in terms of peak height were obtained when a plug of MeOH:ACN (50:50 v/v) was injected for 200 s before the sample injection. With an injection longer than 200 s, the increase of the peak height was not significant and the

peak efficiency deteriorated. Sample injection voltage is an important parameter to be studied in FASI in order to obtain high preconcentration. This study was carried out by monitoring the current until it reached 95 % of the initial current, applying different sample injection voltages between -5 kV and -20 kV. After that, the polarity was switched to positive separation voltage. The results showed a loss of peak efficiency with injection voltages above -5 kV.

As can be observed in Table 3.3.1, the parameters affecting the in-line SPE-CE system are the same as those described in section 3.2. The reverse-phase sorbent Oasis HLB was used to construct the in-line SPE-CE system, as this is the most commonly used sorbent for preconcentrating parabens by off-line SPE with good results of recoveries [2, 3]. Taking into account the pKa values of this group of compounds (i.e. between 8.0 and 9.5), the best results in terms of peak area were obtained when the sample was prepared at a pH value of 3.0, as at this pH, the compounds were retained by the strength of their hydrophobic interactions with the sorbent. After the study of different organic solvents to elute the analytes, ACN provided better results and a volume of 34 nL of this organic solvent allowed the injection of sample for 60 min without reaching the breakthrough volume.

Table 3.3.2. shows the main results for the four preconcentration techniques evaluated: sweeping, LVSS, FASI, and in-line SPE-CE, when standard solutions were analysed. These results show the different preconcentration factors and LODs which can be achieved with the application of these preconcentration strategies. From the electrophoretic preconcentration techniques, the lowest LODs were obtained by FASI (2 ng mL^{-1}), as this strategy was performed with an electrokinetic injection of the analytes, followed by LVSS in which LODs were in the range of 3 ng mL^{-1} to 4 ng mL^{-1}. The highest LODs were achieved by sweeping, ranging from 18 ng mL^{-1} to 27 ng mL^{-1}. These last two preconcentration techniques were performed by a hydrodynamic injection of the sample. For sweeping, lower preconcentration factors (SEF_{LODs} 17- to 29-fold) were obtained and so higher LODs could be attributed to the characteristics of the parabens, which had a moderate interaction with the SDS and were only preconcentrated to a significant extent when a small sample plug (100 s of hydrodynamic injection) was injected, as the injection of larger sample plugs produced a loss of resolution. With in-line SPE-CZE, the SEF_{LODs} obtained were 1,000 or 100 times higher than those of stacking techniques and so the LODs were much lower (between 0.01 ng mL^{-1} and 0.02 ng mL^{-1}). This chromatographic technique allowed the injection of 60 min of sample, a volume of 77.5 µL, which is a large volume compared with the 0.7 µL of sample injected by LVSS. This explains the differences between the LODs and SEF achieved by the electrophoretic

Table 3.3.2 Results obtained using different strategies to decrease the LODs for the analysis of parabens in standard solutions.

CE mode	Strategy to decrease LODs	Capillary	Sample volume	LODs	SEF_{LODs}	Electrophoretic separation time	Total analysis time
CZE	LVSS	50 µm I.D., 53 cm	724 nL (50 mbar 500 s)	3-4 ng mL^{-1}	53-77-fold	9 min	27 min
	FASI	50 µm I.D., 53 cm	-5 kV	2 ng mL^{-1}	105-120-fold	9 min	20 min
	In-line SPE-CE	50 µm I.D., 67 cm	67,500 nL (930 mbar 3600 s)	0.01-0.02 ng mL^{-1}	12,600-18,400-fold	11 min	86 min
MEKC	Sweeping	50 µm I.D., 60 cm	128 nL (50 mbar 100 s)	18-27 ng mL^{-1}	17-29-fold	13 min	21 min

and chromatographic preconcentration techniques studied. Regarding the total analysis time, it can be observed that for the in-line SPE-CE this is considerably longer than for the rest of the evaluated preconcentration techniques. This is attributed to the sample loading time which is 60 min.

In light of the results presented in this study, the developed methodologies (sweeping-MEKC, LVSS-CZE, FASI-CZE, and in-line SPE-CE) present high preconcentration potential which may make these approaches useful strategies for the analysis of different kinds of samples, such as pharmaceutical, cosmetic, food, biological and environmental samples, depending on the concentration expected and the sample matrix.

Regarding the developed in-line SPE-CE method, it is the most sensitive method for the determination of parabens by CE. However, a previous off-line sample preconcentration step would be necessary in order to reach the levels at which these compounds are usually found in environmental waters.

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4. CONCLUSIONS

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The major conclusions that can be drawn from the studies presented in this Doctoral Thesis can be summarized as follows.

1. The sensitivity of CE can generally be considered to be lower than for LC. However, when CE is combined with electrophoretic preconcentration techniques, the sensitivity can improve to achieve similar or even higher values than those obtained by LC.

2. When CE is combined with preconcentration techniques, the developed methodologies for determining PPCPs have a sensitivity of low ng mL^{-1} in the case of electrophoretic preconcentration techniques and low pg mL^{-1} for in-line SPE-CE:

3. When real samples are analysed, such as environmental waters, a pretreatment step, generally based on SPE, is needed in order to clean up the sample and, in some cases, achieve an additional preconcentration factor in order to reach the levels at which these compounds are usually found in this kind of samples.

4. Both sweeping and ASEI-sweeping are adequate techniques for the preconcentration of NSAIDs in river water samples, and only a sample dilution is required.

5. The in-line coupling between SPE and CE has great potential for decreasing the detection limits of CE for the determination of pharmaceutical compounds.

6. The in-line SPE-CE can be applied directly to the analysis of river waters without previous dilution for the determination of pharmaceutical compounds, which demonstrates that this technique is less matrix-dependent than the electrophoretic preconcentration techniques of sweeping and ASEI-sweeping.

7. The coupling of CE to MS, in combination with the in-line SPE-CE preconcentration technique has enabled the determination of four UV-filters at levels of pg mL^{-1} in water samples, resulting in a strategy which reports similar results to chromatographic methods in terms of sensitivity.

8. Oasis MCX sorbent provides good results for in-line SPE-CE for preconcentrating a group of UV-filters. The LODs obtained are the lowest achieved by CE for these compounds.

9. Of the different electrophoretic preconcentration techniques studied in the determination of parabens, LVSS and FASI applied in CZE provided higher preconcentration factors and lower LODs than the sweeping preconcentration technique applied in MEKC.

10. The chromatographic preconcentration technique, in-line SPE-CE, enables the injection of a large sample volume compared to the electrophoretic preconcentration techniques, thereby achieving higher preconcentration factors and lower LODs. This method is the most sensitive for the determination of parabens by CE.

11. The different preconcentration strategies combined with CE (sweeping-MEKC, LVSS-CZE, FASI-CZE and in-line SPE-CZE) evaluated for the determination of parabens provided a different range of focusing. They may therefore be a good starting point for the analysis of different types of samples depending on the expected concentration and on the complexity of the sample matrix.

12. All these results were obtained during this PhD thesis and encourage us to continue studying the preconcentration techniques applied in CE in order to decrease the detection limits. Future research into SPE coupled in-line with CE will focus on using more selective sorbents, such as MIPs, and the combination between different electrophoretic and chromatographic preconcentration techniques, as these factors have considerable potential for improving the sensitivity of electrophoretic analysis.

Annex I. List of abbreviations

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AC	Analyte concentrator
ACN	Acetonitrile
ASEI-sweeping	Anion Selective Exhaustive Injection-Sweeping
BGE	Background electrolyte
BP	Butylparaben
BP-3	Benzophenone-3
BzP	Benzylparaben
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CF-EKS	Counter-flow EKS
CL	Chemiluminescence
co-EOF	co-Electroosmotic flow
CSEI-sweeping	Cation Selective Exhaustive Injection-Sweeping
CSF	Cerebrospinal fluid
CTAB	Cetyltrimethylammonium bromide
CTAC	Cethyltrimethylammonium chloride
CZE	Capillary zone electrophoresis
DETA	Diethylenetriamine
DHB	2,4-dihydroxybenzophenone
DHMB	2,2-dihydroxy-4-methoxybenzophenone
DLLME	Dispersive liquid–liquid microextraction
DTAB	Dodecyltrimethylammonium bromide
DTAC	Dodecyltrimethylammonium chloride
EDTA	Ethylenediamine tetraacetic acid
EKS	Electrokinetic supercharging
EOC	Emerging organic contaminants
EOF	Electroosmotic flow
EP	Ethylparaben
ESI	Electrospray ionization
FAEP	Field-Amplified Sample Injection with Matrix Removal via an EOF Pump
FASI	Field-Amplified Sample Injection
FASS	Field-Amplified Sample Stacking
FESI	Field-Enhanced Sample Injection
FESI-RMM	Field-Enhanced Sample Injection with Reverse Migrating Micelles

FESI-RMP	Field-Enhanced Sample Injection with Reverse Migrating Pseudostationary Phase
GC-MS	Gas chromatography-mass spectrometry
HBP	2,4- dihydroxy-benzophenone
HCB	High-conductivity buffer
HF-LLLME	Hollow fiber membrane liquid-liquid-liquid microextraction
HMBP	2-hydroxy-4-methoxy-benzophenone
HMMA	Hydroxy-3-methoxy-methamphetamine
IA	Immunoaffinity
I.D.	Internal diameter
IL	Ionic liquids
in-line SPE-CE	In-line solid-phase extraction-capillary electrophoresis
IPP	Isopropylparaben
ITP	Isotachophoresis
LC-MS	Liquid chromatography-mass spectrometry
LIF	Laser-induced fluorescence
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LPME	Liquid-phase microextraction
LVSEP	Large Volume Sample Stacking using the EOF pump
LVSS	Large Volume Sample Stacking
LOD	Limit of detection
MEEKC	Microemulsion Electrokinetic Capillary Chromatography
MEKC	Micellar Electrokinetic Capillary Chromatography
MeOH	Methanol
MIP	Molecularly imprinted polymer
MP	Methylparaben
MS	Mass spectrometry
NACE	Nonaqueous capillary electrophoresis
NGS–CE	Non-gel sieving CE
NSAIDs	Non-steroidal anti-inflammatory drugs
NSM	Normal Stacking Mode
PCPs	Personal care products
PLE	Pressurized liquid extraction
PMME	Poly(methacrylic acid-co-ethylene glycol dimethacrylate) monolith microextraction
PP	Propylparaben

PPCPs	Pharmaceutical and personal care products
PMDSA	2-phenylbenzimidazole sulphonic acid
ptITP	Pseudo transient isotachophoresis
PTFE	Polytetrafluoroethylene
PS	Pseudostationary Phase
REPSM	Reversed Electrode Polarity stacking Mode
SBSE	Stir bar sorptive extraction
SD-LLLME	Single-drop liquid-liquid-liquid microextraction
SDS	Sodium dodecyl sulfate
SEI	Selective Exhaustive Injection
SeMet	Selenomethionine
SeOMet	Selenomethionine selenoxide
SPME	Solid-phase microextraction
SPE	Solid-phase extraction
SRMM	Stacking with Reverse Migrating Micelles
SRMM-ASEI	Stacking with Reverse Migrating Micelles-Anion Selective Exhaustive Injection
SRMP	Stacking with Reverse Migrating Pseudostationary Phase
tITP	Transient isotachophoresis
TBAP	Tetrabutylammonium phosphate
TTAB	Tetradecytrimethylammonium bromide
μ_e	Electrophoretic mobility
WWTP	Wastewater treatment plant

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Annex II. Publications

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List of publications derived from this Doctoral Thesis

Maijó, I.; Borrull, F.; Calull, M.; Aguilar, C. *Determination of non-steroidal anti-inflammatory drugs in river water by sweeping-micellar electrokinetic capillary chromatography*. Journal of Liquid Chromatography & Related Technologies (in press) (section 3.1.1.).

Maijó,I.; Borrull, F.; Aguilar, C.; Calull, M. *On-Column preconcentration of anti-inflammatory drugs in river water by anion-selective exhaustive injection-sweeping-MEKC.* Chromatographia 73 (2011) 83-91 (section 3.1.2).

Maijó, I.; Borrull, F.; Calull, M.; Aguilar, C. *An in-line SPE-CE strategy to enhance the sensitivity in C for the determination of pharmaceutical compounds in river water samples.* Electrophoresis 32 (2011), 2114-2122 (section 3.2.1.).

Maijó, I.; Fontanals, N; Borrull, F.; Neusüß, C; Calull, M.; Aguilar, C. *Determination of UV-filters in river water samples by in-line solid-phase extraction-capillary electrophoresis-mass spectrometry.* Electrophoresis (submitted) (section 3.2.2.).

Maijó, I.; Borrull, F.; Calull, M.; Aguilar, C. *Different strategies for the preconcentration and separation of parabens by capillary electrophoresis*. Electrophoresis (accepted) (section 3.3.1.).

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