NOVEL CATION SELECTIVE EXHAUSTIVE INJECTION-SWEEPING PROCEDURE FOR 5-NITROIMIDAZOLE DETERMINATION IN WATERS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY USING DISPERSIVE LIQUID-LIQUID MICROEXTRACTION

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Abstract

A novel method consisting on cation-selective exhaustive injection and sweeping (CSEI-sweeping) as on-line preconcentration followed by a micellar electrokinetic chromatography (MEKC) separation has been developed for the determination of 5-nitroimidazoles (5-NDZ) in environmental waters. Moreover, dispersive liquid-liquid microextraction (DLLME) has been proposed for first time as sample treatment technique prior to CSEI-sweeping-MEKC. DLLME was applied on 5 mL of sample. Dibromomethane (1156 µL) and 2-butanol (1363 µL) were employed as extractant and dispersive solvents, respectively. Salting-out effect was achieved by the addition of 16 % (w/v) NaCl to the samples. After DLLME and organic solvent evaporation, the residue was redissolved in a low conductivity solvent (5 mM phosphoric acid with 5% of methanol) and electrokinetically injected at 9.8 kV for 632 s in a bare fused-silica capillary (57.2 cm, 50 µm I.D.). Prior to the injection, the capillary was rinsed with 50 mM phosphate buffer pH 2.5, followed by a plug of a higher conductivity buffer (100 mM phosphate pH 2.5, 50 mbar, 264 s) and a plug of water (50 mbar, 2 s). Separation was carried out applying -30 kV at 20 ºC in 44 mM phosphate buffer pH 2.5, containing 8% tetrahydrofuran and 123 mM sodium dodecyl sulfate. Analytical signals were monitored at 276 nm. Validation was performed in river and well waters, obtaining satisfactory results in terms of linearity, precision (% RSD generally lower than 10%) and trueness (recoveries higher than 70% in almost all cases). LODs ranged from 0.61 to 2.44 ng/mL. The combination of this microextraction technique with the proposed capillary electrophoresis methodology supposes a simple, sensitive and cheap alternative for 5-NDZ analyses, in accordance with the aims of green chemistry.

1. Introduction

Metronidazole (MNZ) is an antibiotic which belongs to 5-nitroimidazole (5-NDZ) family. It is widely used in humans for treating diseases due to anaerobia microbes [1]. It is considered an essential antibiotic according to World Health Organization (WHO) [2]. Other drugs from the same family, such as tinidazole (TNZ), ornidazole (ORZ), carnidazole (CRZ), ipronidazole (IPZ), secnidazole (SCZ) and ternidazole (TRZ) have been also considered in the current work. The chemical structures of the selected compounds are shown in Figure 1. In spite of the effectiveness of 5-NDZs as antibacterial and antiprotozoal agents, their employment in the veterinary practice is restricted. Some reports attribute mutagenic, carcinogenic and genotoxic properties to 5-NDZs [3-4], and therefore their application have been banned in animals intended for human consumption in European Union (EU), United States (US) and China [5-7]. On the other hand, 5-NDZs possess high polarity and low biodegradability, which involves high bioaccumulation levels and, consequently, ecotoxicity [8]. The presence of antibiotics such as 5-NDZs in sewage water has been already reported [9], even though a few number of papers have been focused on their removal from wastewater treatment plants [10,11]. For all these reasons, 5-NDZ drugs are considered as emerging water micropollutants. The environmental risks caused by drugs have been studied in the past decade [12], however more research in this area is still required, including updated reports about their presence and levels in ecosystems. Thus, analytical methodology for detection and quantification of these residues in the environment is highly needed in order to evaluate the exposition of the environment to antibiotics and their risks [13].

Several methods have been proposed for 5-NDZ determination [14-17], however, applications to environmental water samples are reduced [18-20]. New contributions about 5-NDZ determination in aquatic environmental samples are desired, taking into account that their presence has been already reported in natural waters [21]. Traditionally, liquid chromatography (LC) coupled to mass spectrometry (MS) has been the most popular choice for monitoring 5-NDZ residues [22-23]. Capillary electrophoresis (CE) [24] or gas chromatography (GC) [25] are among the proposed alternatives to LC. Low solvent consumption, short analysis time and high efficiency are CE characteristics; however its use is limited due mainly to the poor sensitivity, especially when it is coupled to ultraviolet (UV) detection. In order to overcome this disadvantage, different preconcentration strategies have been developed, including on-line (sample stacking techniques) [26], in-line [27] and off-line (through sample pretreatment) procedures[28]. The most common on-line preconcentration methodologies include: acetonitrile

stacking [29], field-amplified sample stacking (FASS) and field-amplified sample injection (FASI) [30], sweeping [31], dynamic pH junction [32] and isotachophoretic stacking [33], although others less known strategies offer even higher sensitivity enhancement factors (SEF).

A decade ago, Quirino et al. proposed a novel on-line preconcentracion technique, based on cation-selective exhaustive injection and sweeping (CSEI-sweeping). It combines two on-line preconcentration techniques: field-enhanced sample injection (FESI) and sweeping. This combination allows achieving enhancement factors from a thousand- to almost a million-fold in relation to conventional CE [34]. CSEI-sweeping involves electrokinetic injection (FESI) of a high amount of charged cationic compounds, creating long analyte zones in the capillary with higher concentration than in the original sample solution [35]. After sample injection, background electrolyte (BGE) vials containing micelles are placed at both ends of the capillary and negative voltage is applied. Micelles focused the analytes in narrow bands by sweeping, and they are consequently separated by conventional micellar electrokinetic chromatography (MEKC). A procedure scheme is shown in Figure 2. To achieve FESI, analytes must be dissolved in a low-conductivity medium, which is not so obvious, especially for complex real samples showing high or moderate salinity. In such cases, a proper sample pretreatment is needed in order to reduce sample conductivity. Another drawback inherent to CSEI-sweeping-MEKC is the run time since it requires longer analysis times compared to conventional CE, considering capillary conditioning, injection and separation time [34]. However, most commercial CE instruments allow the automation of capillary preconditioning, sample injection and CE separation. Currently CSEI-sweeping-MEKC is a quite novel technique since it has not been much exploited yet, however it has been successfully employed e.g. for thedetermination of drugs of abuse in urine [36-37]; methadone in serum [38]; herbicides in water samples [39]; and melamine and cyromazine in milk [40].

Solid phase extraction (SPE) has demonstrated to be a good option for sample clean-up prior to CSEI-sweeping-MEKC [41-42]. Other techniques have been also employed, such as cloud point extraction [43] or hollow fiber based liquid-liquid-liquid microextraction (HF-LLME) [44]. Dispersive liquid-liquid microextraction (DLLME) is a novel microextraction method introduced by Rezaee et al. [45]. Due to its simplicity, low cost and low solvent consumption, it has been very popular as sample pretreatment during last decade. DLLME consists on a quick injection of an organic solvent mixture (water-immiscible organic extractive solvent plus water-miscible organic dispersive solvent) into an aqueous sample causing a cloudy dispersion. Dispersive solvent enhances the exchange surface between the extractive solvent and the

aqueous sample matrix, assisting the analyte extraction. It is a miniaturized sample treatment technique which has found its major application field in water analyses with satisfactory results [46]. To the best of our knowledge, DLLME has never been employed for sample treatment prior to a CSEI-sweeping-MEKC procedure.

In this work, a novel CSEI-sweeping-MEKC method has been developed for 5-NDZ determination in water samples. In addition, DLLME has been successfully coupled to CSEI-sweeping-MEKC, sinceDLLME yields low conductivity extracts compatible with the requirements of FESI. The combination of DLLME as sample treatment and CE is a green alternative for 5-NDZ analyses, considering the low consumption of reagents and sample and the low impact of the buffers employed as separation media. Also DLLME represents a quick procedure which compensates the time needed for the on-line preconcentration and separation method.

2. Materials and Methods

2.1 Chemicals and reagents

All reagents were analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, US) was used throughout the work. Sodium chloride (NaCl), sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4) and orthophosphoric acid (H_3PO_4) (85%) were obtained from Panreac-Química (Madrid, Spain); methanol (MeOH) and 2-butanol were supplied by VWR International (West Chester, PA, US). Acetonitrile (MeCN), dibromomethane, isopropanol, sodium dodecyl sulfate (SDS) and oxalic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98-100%) and tetrahydrofuran (THF) were supplied by Merck (Darmstadt, Germany).

Analytical standards of CRZ ([2-(2-methyl-5-nitro-imidazol-1-yl)ethyl]thiocarbamic acid o-methyl ester), ORZ (1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole), MNZ (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) and TNZ (1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole) were supplied by Sigma-Aldrich (St. Louis, MO, US), while IPZ (2-isopropyl-1-methyl-5-nitroimidazole), SCZ (α,2-dimethyl-5-nitro-1H-imidazole-1-ethanol hemihydrate) and TRZ (1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole) hydrochloride were purchased from Witega (Berlin, Germany).

0.2 µm nylon membrane filters (Pall Corp, MI, US) were used for sample filtration.

2.2 Standard solutions preparation

142 Individual stock standard solutions of every 5-NDZ containing 1.00 g/L were prepared by
143 dissolving each pure compound in MeCN. These solutions were stored in dark bottles at -20°C
144 and equilibrated to room temperature before use. They were stable for at least six months.
145 Intermediate standard solutions of 2.00 mg/L of each 5-NDZ, except for TNZ (4.01 mg/L), were
146 prepared by mixing aliquots of each individual stock standard solution and diluting with MeCN.
147 These solutions were stored at 4°C and exposure to direct light was avoided. They were stable
148 for at least three months.
149 Fresh working standard solutions of lower concentrations were daily prepared in injection
150 medium.
151
152 ***2.3 Instrumentation and software***
153 CE experiments were carried out with an Agilent 7100 CE System (Agilent Technologies,
154 Waldbron, Germany) equipped with a diode-array detector. Data were collected using the
155 software supplied with the HP ChemStation (Version B.02.01). Separations were performed in a
156 57.2 cm x 50 µm internal diameter (i.d.), uncoated fused-silica capillary with an optical path
157 length of 150 µm (bubble cell capillary from Agilent Technologies) and an effective length of 48.7
158 cm.
159 A pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit,
160 a centrifuge (Universal 320 model from Hettich, Leipzig, Germany), an evaporator with nitrogen
161 (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from
162 Scientific Industries, Bohemia, USA) were also used.
163 UNSCRAMBLER® v 9.8 software [47] was used for evaluating the obtained results.
164
165 ***2.4 Preparation of samples***
166 Natural water samples from different sources were considered in this study. River water
167 samples were collected from Riofrío river (Riofrío, Granada, Spain), being the sampling point
168 located after a fish farm drain, and from Genil urban river (Granada, Spain). Besides, water
169 samples from a well placed in a cattle area (La Serena, Badajoz, Spain) were also studied. Water
170 samples were kept at 4°C and equilibrated to room temperature before analysis.
171 The employed DLLME procedure for water sample treatment has been previously
172 reported by our group [18]. The sample treatment was applied to 5.00 mL aliquots of each water
173 sample contained in a 15 mL centrifuge tube with a conical bottom. Analyte extraction was
174 assisted by a salting out effect, adding 0.800 g of NaCl (16% w/v) to each sample. Salt was

dissolved in water samples by vortexing before carrying out the extraction. 5-NDZ extraction took place through the quick injection of an organic solvent mixture into the water sample consisting on 1156 µL of dibromomethane as extraction solvent and 1363 µL of 2-butanol as dispersive solvent. Injection was carried out with a syringe coupled to a needle with a flat point and it caused a cloudy solution. Afterwards, the sample tube was vortexed for 30 s and it was centrifuged for 5 min at 9000 rpm. Phase separation occurred, obtaining the organic layer as sediment. It was carefully collected with a syringe coupled to a needle with a flat point and it was placed into a glass vial. It is important to avoid the collection of aqueous phase because of its high salt content. After the organic phase collection, it was evaporated to dryness under gentle nitrogen current and it was redissolved with 1.20 mL of an acidic aqueous solution (5.00 mM of orthophosphoric acid containing 5% of MeOH), vortexed for 2 min and filtered. Finally, 1.00 mL of the extract was analysed.

2.5 CSEI-sweeping-MEKC procedure

Water sample analysis was performed in an uncoated fused-silica capillary (57.2 cm x 50 µm i.d). A new capillary was conditioned for the first time with NaOH 1 M for 15 min at 1 bar of pressure and 20°C. Afterwards and under the same conditions, capillary was rinsed with ultrapure water for 5 min and with a low conductivity buffer (LCB) for 15 min. LCB consisted of a 50 mM phosphate buffer (NaH_2PO_4/H_3PO_4, pH = 2.5). Every day, capillary was washed with LCB for 15 min at 1 bar of pressure and 20°C. At the end of each day, capillary was washed with deionized water for 5 min at 5 bars followed by air flush for 5 min at 5 bars and at working temperature. Between runs, capillary was rinsed with NaOH 0.1 M at 3 bars for 2 min followed by ultrapure water at 3 bars for 0.5 min and lastly it was rinsed with LCB at 3 bars for 3 min. Subsequently, capillary was flushed with a high conductivity buffer (HCB) for 264 s at 50 mbars (capillary was filled a 31.5% of its total length) and 20°C. HCB consisted of a 100 mM phosphate buffer (NaH_2PO_4/H_3PO_4, pH = 2.5). Afterwards, inlet electrode was submerged into a vial containing ultrapure water for 5 s in order to wash it. Finally, an ultrapure water plug was injected into the capillary by applying a pressure of 50 mbar for 2 s. After capillary conditioning, samples were electrokinetically injected at 9.8 kV (normal mode) for 632 s. Electrophoretic separation was performed under a voltage of -30 kV programing a voltage ramp from 0 to -30 kV for 0.5 min at the beginning of the run. Separation temperature was 20°C. Background electrolyte (BGE) consisted of a phosphate buffer (44.0 mM, pH = 2.5) containing 123 mM of SDS and 8.00% of tetrahydrofuran (THF). UV detection was carried out at 276 nm, except for CRZ (244 nm).

3. Results and discussion

3.1. Optimization of CSEI-sweeping-MEKC method

Initially, separation was performed in a 48.5 cm x 50 μm capillary. It was rinsed with LCB solution (25 mM phosphate buffer NaH_2PO_4/H_3PO_4, pH = 2.5) at 3 bars for 3 min at working temperature. Afterwards, HCB solution (150 mM phosphate buffer NaH_2PO_4/H_3PO_4, pH = 2.5) was flushed for 150 s into the capillary at 50 mbars and at work temperature. Inlet electrode was washed with ultrapure water for 5 s. A water plug (1 s at 50 mbars) was introduced prior to the sample injection. The sample, dissolved in 3 mM H_3PO_4 solution, was injected for 600 s at 10 kV. 5-NDZ separation was performed at -30 kV and 20°C, employing a buffer solution of 20 mM phosphate (pH = 2.5) containing 150 mM of SDS as BGE. Analytical signals were monitored at 276 nm during the method optimization. Standard solutions of 70 ng/mL of each studied analyte (IPZ, ORZ, SCZ, TRZ and MNZ), except for CRZ (210 ng/mL), were used in this study.

Variables related to the CSEI-sweeping-MEKC methodology have been divided in three groups for their optimization: parameters affecting separation (buffer concentration, organic percentage in the buffer and surfactant concentration); chemical variables related to the capillary conditioning (concentration of LCB, concentration of HBC and capillary length filled with HCB); and instrumental variables related to the injection (voltage and injection time). These groups of variables were optimized through experimental designs in order to consider their interactions. Other parameters, such as separation pH, separation buffer nature, injection media, buffer concentration in the outlet vial during the sample injection, influence of a water plug before the sample injection and separation voltage were optimized univariately.

3.1.1. Separation buffer pH and nature

Separation buffer pH was studied in a narrow range (between 2 and 3). At lower pH values, electroosmotic flow (EOF) was very low and consequently worse analyte stacking effect was observed. At higher pH values, a stronger EOF was produced, and as consequence, EOF was higher than the electrophoretic velocity of the micelles. It resulted in analyte migration towards the cathode instead of from the inlet vial (cathode) to the outlet vial (anode). Considering the evaluated range, longer analysis times were observed at pH 3, while poorer stacking effect was observed at pH 2. Thus, separation pH was fixed to 2.5.

Different buffer natures as phosphate, oxalate and formate were studied. All the buffers were prepared at a concentration of 20 mM. Oxalate and formate buffers were prepared from their respective acids and adjusting the pH with NaOH, while phosphate buffer was prepared by mixing NaH_2PO_4 and H_3PO_4. Phosphate buffer was chosen as the optimum because of the oxalate buffer showed bad reproducibility while formate buffer gave the lowest peak heights.

In order to increase peak resolution, different organic solvents were evaluated as modifiers of the separation buffer. (10% of MeCN, MeOH, isopropanol and THF). In all cases, similar peak resolution was shown and the only improvement observed was in terms of peak height. Signal increase was more appreciable when THF was added to the separation buffer.

Negatively charged micelles are required in CSEI-sweeping methodologies. In this work, SDS has been selected, considering it has been the most employed surfactant in the application of this technique [36-40].

3.1.2. Separation buffer concentration

A central composite design was employed for the multivariate optimization of surfactant concentration, phosphate buffer concentration and percentage of THF contained in BGE. Ranges for each parameter were established as follows: from 10.23 to 70.77 mM for phosphate buffer concentration, from 49.32 mM to 200.7 mM for SDS concentration and between 0 and 15.07% for THF volume contained in the separation buffer. Equation 1 represents the employed response function (R.F.). Parameters in R.F. have been normalized respect to the maximum value shown in the experimental set. The selected R.F. considers the most critical parameters for the proposed 5-NDZ separation. Lowest resolution was obtained between SCZ and TRZ peaks, so the normalized resolution ($R_{norm.}^{Peaks\ SCZ-TRZ}$) among both peaks was included in R.F. Other terms such as normalized CRZ and MNZ theoretical plates are also included in the R.F. (equation 1). It is due because preliminary studies indicated that CRZ presented the lowest sensitivity while MNZ is the most important 5-NDZ drug due to its wide used.

$$R.F. = Plates_{norm.}^{CRZ} + Plates_{norm.}^{MNZ} + R_{norm.}^{Peaks\ SCZ-TRZ} \quad \textit{Equation 1.}$$

In the corresponding analysis of variance (ANOVA), a second-degree quadratic model was assumed. Lack-of-fit was no significant at a confident level of 95.0% (*p*-value > 0.05). In this case, interaction between THF percentage and SDS concentration was significant (*p*-value = 0.0131) as well as the quadratic interactions (*p*-value > 0.05). The optimum values for the studied variables were: 44 mM phosphate buffer, 123 mM SDS and 8% THF. These values were established for further experiments.

3.1.3. Separation voltage and temperature

Separation was carried out at negative polarity. Voltage values from -25 kV to -30 kV were evaluated. Values below -30 kV provided longer analysis times without any improvement on peak resolution. According to that, -30 kV was established as optimum. Due to the high voltage employed, low separation temperature was desired. Temperature was set at 20°C in order to avoid capillary heating because of Joule effect.

3.1.4. Water plug prior sample injection

Some reports attribute an improvement on sensitivity to a water plug introduced into the capillary prior to sample injection. Before sample injection, water plugs injected under pressure at 50 mbars for 1, 2, 3 and 4 s were tested. In this case, a water plug did not involve an increase on sensitivity so it was suggested to consider a water plug for 2 s prior to sample injection because it produced a better injection reproducibility [48].

3.1.5. Chemical parameters of CSEI

Phosphate buffer was selected as LCB and HCB solution. Concentration of both solutions, together with the capillary length filled with HCB solution, were evaluated by a central composite design. Studied experimental domains ranged from 9.82 to 50.18 mM for LCB concentration, from 74.43 to 200.57 mM for HCB concentration, and finally, the injection time of HCB solution (under a pressure of 50 mbars) was studied from 60.18 to 241.82 s. The considered R.F is indicated in equation 2.

$$R.F. = \frac{H^{MNZ}}{1+|1-Sym^{MNZ}|} \quad \textit{Equation 2.}$$

In this R.F., only the symmetry (Sym^{MNZ}) and height (H^{MNZ}) of MNZ peak have been included. According to that, analyte stacking into the capillary has been evaluated considering MNZ peak as representative. A higher stacking effect involves higher sensitivity in terms of peak height, without any losing of peak symmetry. In the proposed experimental design, lack of fit was no significant ($p > 0.05$) at a confident level of 95.0%. The obtained optimum values from the surface response were 50 mM phosphate in LCB, 100 mM phosphate in HCB and 190 s of HCB injection time (capillary was filled a 31.5% of its total length). The influence of LCB and HCB concentrations on R.F. was found to be significant at a confidence level of 95 % (*p*-value =

0.0030 and *p*-value = 0.0163, respectively) as well as quadratic HCB concentration interaction (*p*-value = 0.0344).

3.1.6. Instrumental parameters for FESI performance

A central composite design was again employed to optimize voltage and injection time. Variables were continuously studied from 5 to 15 kV for the injection voltage and from 5 to 15 min for the injection time. Equation 3 was established as R.F. in order to improve CRZ and MNZ peak signals. For that reason, CRZ and MNZ normalized peak height ($[H_{norm.}^{CRZ} + H_{norm.}^{MNZ}]$) were included as R.F. terms. Because of shapeless peaks were shown under some of the employed injection conditions, a term that represents the number of symmetrical peaks ($n_{gaussians\ integrable\ peaks}$) was also included in the R.F. The number of analytes ($n_{analytes}$) studied in this experimental design was six.

$$R.F. = [H_{norm.}^{CRZ} + H_{norm.}^{MNZ}] \cdot \frac{n_{gaussian\ defined\ peaks}}{n_{analytes}} \quad \textit{Equation 3.}$$

Lack-of-fit was no significant at a confident level of 95.0%. The maximum of the response surface corresponds to an injection voltage of 9.8 kV and an injection time of 10.53 min. The response surface for the described experimental design can be found in Figure 3.

3.1.7. Sample injection medium

Low conductivity sample matrices benefit the analyte injection into the capillary by FESI, obtaining an important signal enhancement. However, the presence of a certain amount of protons in sample matrices is needed for obtaining charged analytes, and consequently obtaining a satisfactory injection by FESI. The needed proton concentration is determined by the analyte pK_a values. Because of that, a phosphoric acid solution was proposed as injection media. An organic solvent was added to the injection solution in order to decrease its conductivity. MeCN, MeOH and THF at concentrations of 10% (v/v) were evaluated. Although peak signal improvement was expected, results did not show this behaviour. It could be due to the increase of the injection solution viscosity when an organic solvent is added, involving lower analyte mobility and a lower amount of injected analytes, resulting in lower peak signals. However, the addition of organic additives was considered to guarantee its low conductivity and a satisfactory sample injection as well as better peak symmetries. Better peak symmetries were observed when MeOH was employed so its percentage in the injection buffer was evaluated in the range between 2 and 20%. Slight differences were noticed in these experiments, so 5% was considered as optimum, in

order to avoid a high solution viscosity. Acid concentration was studied in the range between 1 and 10 mM. Signals did not show any variation when acid concentration was higher than 3 mM. At lower concentrations, peak signal depended on each analyte. In this case, a concentration of 5 mM was chosen as optimum because higher concentrations could result in high conductivity samples while lower concentration could be detrimental for charging the analytes.

In order to obtain satisfactory reproducibility for sample injection, the nature of the solution placed in the outlet vial during sample injection to close the circuit was evaluated. Better reproducibility was showed when HCB was used as solution contained into the outlet vial.

Figure 4 shows an electropherogram of standard samples analysed under the proposed CSEI-sweeping-MEKC method considering the optimum conditions.

3.2. Method characterization for water samples

For the first time, DLLME is proposed as sample treatment coupled to CSEI-sweeping-MEKC. In this work, DLLME was applied to different water matrices using conditions previously established [18]. As it is usual, separation method was optimized employing standard solutions of the analytes. However, for the method characterization with spiked matrices, analyte behaviours showed slight variations in terms of peak migration times. Lower peak resolution was shown for analytes in water samples after DLLME treatment, so a longer capillary was used. A capillary of 57.2 cm length was employed for method characterization on real water samples. Considering that, the capillary must be filled with HCB solution for 264 s, reaching the same experimental conditions (31.5 % of the total length filled with HCB) that those proposed for this novel CSEI-sweeping-MEKC strategy.

ORZ determination in water samples was not possible due to the presence of matrix interference at the same migration time. However, new sample injection conditions compared to preliminary ones led to evaluate a new 5-NDZ. TNZ presents lowest pK_a, being injected under the actual injection conditions, although it was not injected under the previously optimized conditions.

All 5-NDZs were monitored at 276 nm, except CRZ which presents at maximum UV absorption at 244 nm.

3.2.1. Calibration curves and analytical performance characteristics

Matrix-matched calibration curves for the studied analytes (CRZ, IPZ, SCZ, TRZ, MNZ and TNZ) were established using river water from Riofrío river (Granada, Spain) as representative matrix to characterize the present method. River water samples were spiked at 2.00, 4.00, 8.00, 14.0 and 20.0 ng/mL for all analytes except to TNZ (4.01, 8.02, 16.0, 28.1 and 40.1 ng/mL). Five aqueous samples were spiked at the same concentration level. They were processed following the previously described procedure and injected and analysed according to the developed CSEI-Sweeping-MEKC methodology. Peak area was considered as a function of the analyte concentration on the sample. A blank sample was treated and no interferences were co-migrating with any 5-NDZ. Figure 5 shows an electropherogram of a spiked river water sample at 14.0 ng/mL for each compound except for TNZ (28.1 ng/mL).

Statistical parameters calculated by least-square regression and the performance characteristics of the DLLME-CSEI-sweeping-MECK-UV method for water samples are shown in Table 1. Limits of detection (LODs) were calculated as 3×S/N as well as by Long and Winefordner [49] and Clayton criteria [50]. Quantification limits (LOQs) were calculated as 10×S/N. Taking into account that the off-line preconcentration factor due to the DLLME applied as sample treatment resulted in only 4.2 times, all 5-NDZ compounds were able to be quantified at the low ppb levels using the proposed methdology, in spite of the poor sensitivity attributed to CE-UV methods.

3.2.2. Precision study

The precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed DLLME-MEKC-sweeping-MEKC-UV method to water from Riofrío river spiked at three different concentration levels (4.00, 8.00 and 20.0 ng/mL) for all analytes (CRZ, IPZ, SCZ, TRZ and MNZ) except for TNZ (8.02, 16.0 and 40.1 ng/mL). Repeatability was studied for seven samples (experimental replicates) while intermediate precision was assessed for five consecutive days analysing one sample each day. No instrumental replicates for samples were considered. FESI involves an exhaustive injection and an important depletion is produced in the vial as a consequence of a single injection [34]. The results expressed as RSD (%) of the peak areas are summarized in Table 2. Satisfactory results were obtained in terms of precision, being RSD (%) lower than 10% in almost cases for all 5-NDZ drugs.

3.3.3. Trueness assessment

Trueness assays were carried out over different spiked water samples. Water samples from Riofrío river (Granada, Spain) and Genil river (Granada, Spain) and well water samples (Badajoz, Spain) were evaluated. Water samples were spiked at three different concentration levels (4.00, 8.00 and 20.0 ng/mL) for all analytes (CRZ, IPZ, SCZ, TRZ and MNZ) except to TNZ (8.02, 16.0 and 40.1 ng/mL). Seven samples from Riofrío river for each concentration level were treated following the proposed method. In the case of Genil river water samples and well water samples, five samples were required for each evaluated concentration level. A blank of each type of water was processed, and an interference associated to the matrix was co-migrating with CRZ in the case of Genil river and well water samples. Recovery values could not be established for this compound in the above-mentioned matrices. For the rest of 5-NDZ drugs, the obtained results are presented on Table 3. In general, recoveries over 70% were obtained.

4. Conclusions

In conclusion, a novel CSEI-sweeping-MEKC has been developed for 5-NDZ determination. Several of the involved chemical and instrumental variables have been chemometrically optimized through experimental designs.

For the first time, DLLME has been coupled to this CE-based methodology. DLLME is a miniaturized sample clean-up treatment with a low solvent consumption. Low conductivity samples were obtained after the DLLME procedure, accomplishing with the proposed CE requirements. So, DLLME has shown to be suitable as sample treatment prior to CSEI-sweeping-MEKC analyses. The proposed DLLME/CSEI-sweeping-MEKC has been successfully applied to the determination of 5-NDZ residues in environmental waters. River and well waters have been also tested. In spite of low CE-UV sensitivity, the developed CSEI-sweeping-MEKC method results in a high on-line preconcentration factor, reaching detection limits lower than 2.44 ng/mL for all the studied analytes. Satisfactory results have been achieved for repeatability and intermediate precision studies, obtaining RSD lower than 10 % in most cases. The combination of DLLME with the evaluated CE technique supposes a cheap and green alternative for monitoring 5-NDZ residues in waters.

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Figure captions

Figure 1.- Chemical structures of evaluated 5-NDZ compounds. *pKa values have been obtained from Scifinder ® database.

Figure 2.- CSEI-Sweeping-MEKC procedure scheme. Adapted from reference [34]. Steps: A, capillary is rinsed with a low conductivity buffer (LCB), followed by a plug of a higher conductivity buffer (HCB) and a water plug; B, electrokinetic injection at positive polarity, being cationic analytes stacked at the interface between the water zone and the HCB zone; C, cationic analytes are stacked at the HCB zone because of the long injection, but not at the water or matrix zone; D, background electrolyte is placed in both ends of the capillary and a negative voltage is applied; E, ordinary MEKC separation takes place.

Figure 3.- Estimated response surface for voltage and injection time associated to FESI.

Figure 4.- Electropherograms of standard analyte solutions obtained by the proposed CSEI-Sweeping-MEKC procedure using an extended path capillary (48.5 cm x 50 μm). Signals are monitored at 276 nm. A. 10 ng/mL of each analyte. B. 75 ng/mL of each analyte. Peaks (1) CRZ, (2) IPZ, (3) ORZ, (4) SCZ, (5) TRZ, (6) MNZ.

Figure 5.- Electropherogram of a spiked river water sample at 14 ng/mL of each compound, except for TNZ concentration (28.1 ng/mL) . Peaks (1) CRZ, (2) IPZ, (3) SCZ, (4) TRZ, (5) MNZ, (6) TNZ. Separation current is showed.

Table 1. Statistical and performance characteristics of the DLLME-CSEI-sweeping-MEKC-UV method for 5-NDZ determination in river water sample.

Analyte	Linear range (ng/mL)	R^2	Analytical resolution (γ^{-1}) (ng/mL in sample)	LOD (ng/mL in sample)			LOQ (ng/mL in sample)
				S/N = 3	Long	Clayton	S/N = 10
CRZ	4.38-20.0	0.980	1.01	1.31	1.04	2.24	4.38
IPZ	2.05-20.0	0.998	0.23	0.61	0.23	0.57	2.05
SCZ	4.60-20.0	0.995	0.42	1.38	0.41	1.00	4.60
TRZ	2.10-20.0	0.995	0.46	0.63	0.44	1.05	2.10
MNZ	2.79-20.0	0.985	0.72	0.84	0.69	1.69	2.79
TNZ	8.14-40.1	0.980	1.86	2.44	1.87	4.26	8.14

Table 2.- Precision study for the proposed method for the determination of 5-NDZs in river water sample.

	Repeatability (% RSD; n = 7)			Intermediate precision (% RSD; n = 5)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
CRZ	7.60	8.13	6.43	23.6	8.13	9.86
IPZ	15.9	11.3	4.32	24.3	3.15	8.40
SCZ	15.5	6.46	7.64	11.4	10.9	7.85
TRZ	4.88	7.70	6.91	10.7	3.80	5.00
MNZ	4.32	7.12	7.26	6.93	6.45	4.92
TNZ	3.91	7.00	9.80	4.13	3.80	5.58

Level 1: 4.00 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; 8.02 ng/mL for TNZ.

Level 2: 8.00 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; 16.0 ng/mL for TNZ.

Level 3: 20.0 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; 40.1 ng/mL for TNZ.

Table 3.- Recovery (R) percentages for 5-NDZs in river and well water samples using DLLME as sample treatment.

	Riofrío river water (% R; n = 7)			Genil river water (% R; n = 5)			La Serena well water (% R; n = 5)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
CRZ	83.3	73.0	82.8	-	-	-	-	-	-
IPZ	87.9	95.1	93.2	72.0	86.1	73.1	66.0	68.5	63.7
SCZ	68.6	73.6	97.2	78.9	91.3	82.0	84.9	83.1	74.0
TRZ	85.3	96.9	93.8	90.5	76.7	76.9	74.0	72.9	69.3
MNZ	76.9	83.8	72.0	80.3	81.5	74.6	68.7	60.9	67.3
TNZ	85.1	91.8	100.0	87.6	87.1	88.1	81.9	73.2	79.3

Level 1: 4.00 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; and 8.02 ng/mL for TNZ.

Level 2: 8.00 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; and 16.0 ng/mL for TNZ.

Level 3: 20.0 ng/mL for CRZ, IPZ, SCZ, TRZ and MNZ; and 40.1 ng/mL for TNZ.

Figure 1

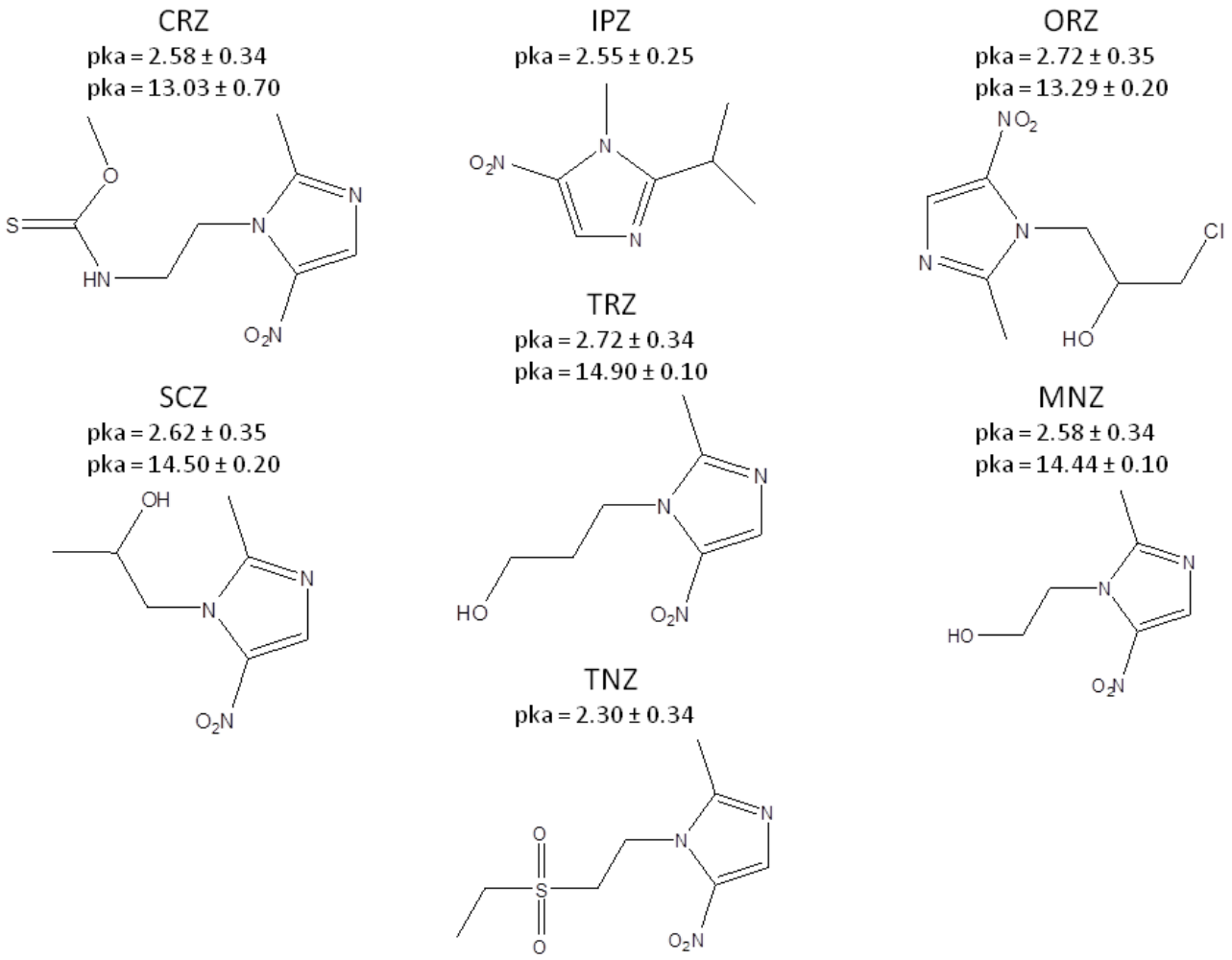
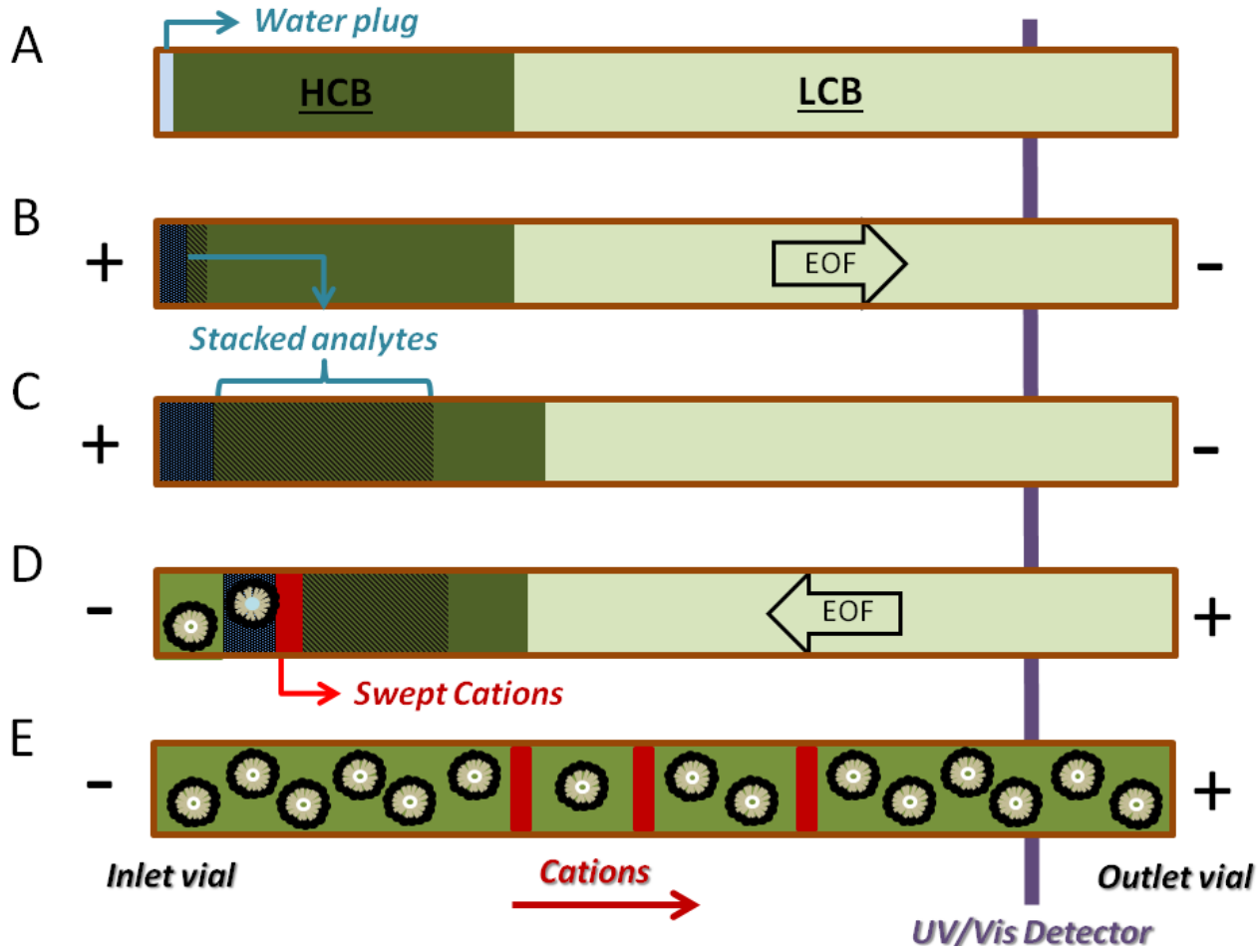

CRZ
pka = 2.58 ± 0.34
pka = 13.03 ± 0.70
IPZ
pka = 2.55 ± 0.25
ORZ
pka = 2.72 ± 0.35
pka = 13.29 ± 0.20
TRZ
pka = 2.72 ± 0.34
pka = 14.90 ± 0.10
SCZ
pka = 2.62 ± 0.35
pka = 14.50 ± 0.20
MNZ
pka = 2.58 ± 0.34
pka = 14.44 ± 0.10
TNZ
pka = 2.30 ± 0.34

Figure 2


A
Water plug
HCB
LCB
B
+
EOF
-
Stacked analytes
C
+
-
D
-
EOF
+
Swept Cations
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+
Inlet vial
Cations
Outlet vial
UV/Vis Detector

Figure 3

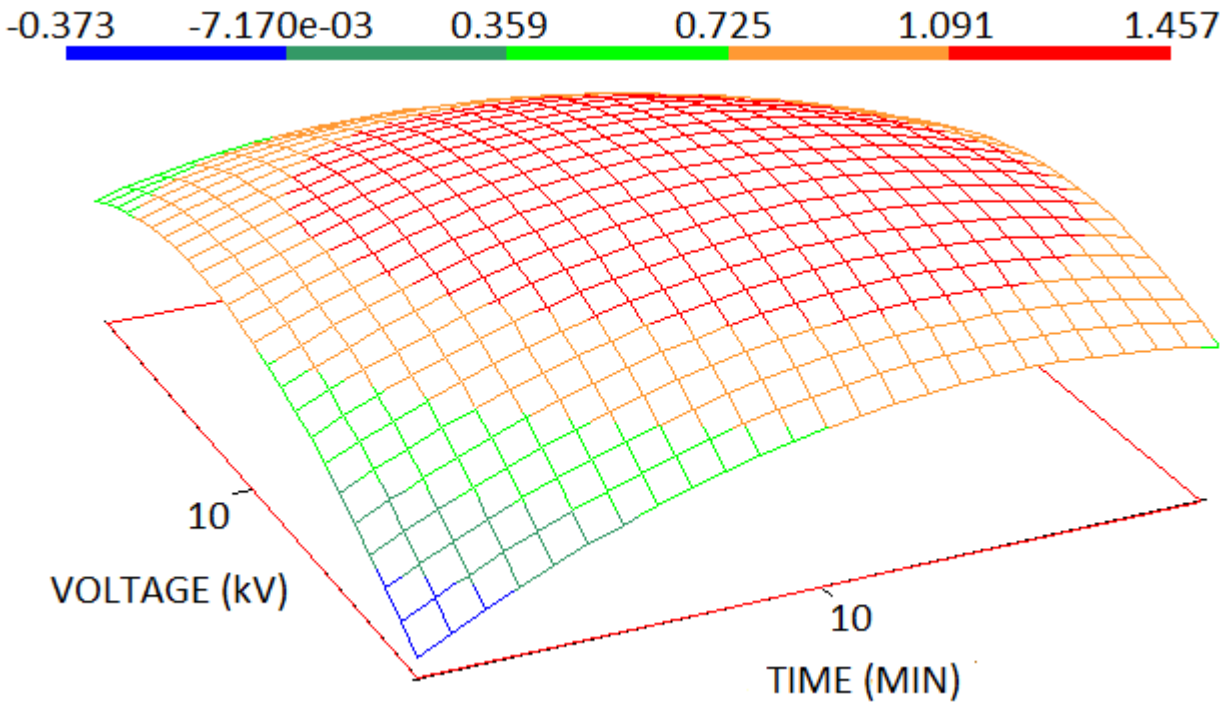


Figure 4.

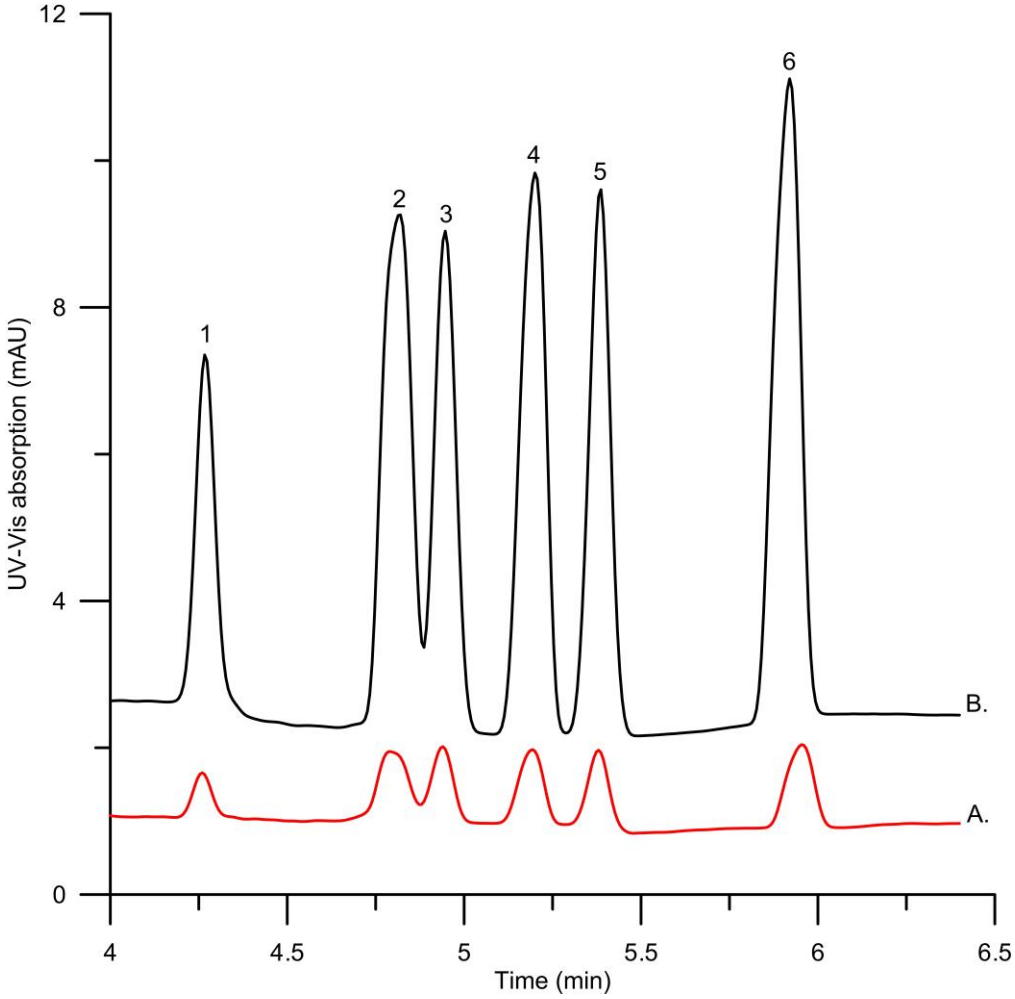
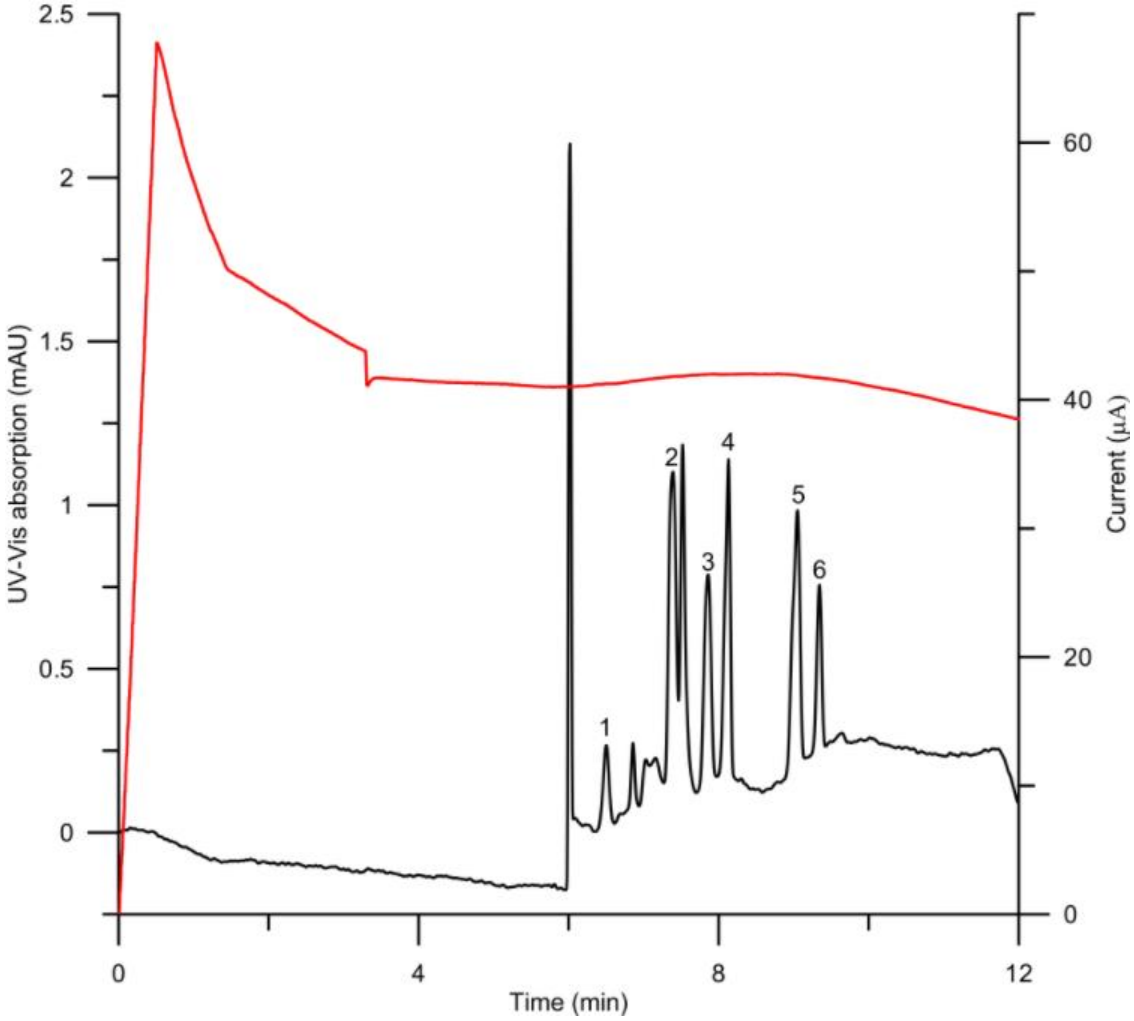


Figure 5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1.5
1
0.5
0
UV-Vis absorption (mAU)
60
40
20
0
Current (μA)
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4
8
12
Time (min)
1
2
3
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5
6