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Profiling cocaine residues and pyrolytic products in wastewater by mixed-mode liquid chromatography-tandem mass spectrometry

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Abstract

This work provides a new analytical method for the determination of cocaine, its metabolites benzoylecgonine and cocaethylene, the pyrolytic products anhydroecgonine and anhydroecgonine methyl ester and the pharmaceutical levamisole in wastewater. Samples were solid-phase extracted and extracts analyzed by liquid chromatography-tandem mass spectrometry using, for the first time in the illicit drug field, a stationary phase that combines reversed-phase and weak cation-exchange functionalities. The overall method performance was satisfactory, with limits of detection below 1 ng/L, relative standard deviations below 21% and percentages of recovery between 93% and 121%. Analysis of 24-h composite raw wastewater samples collected in Santiago de Compostela (Spain) and Brasilia (Brazil) highlighted benzoylecgonine as the compound showing the highest population-normalized mass loads (300-1000 mg/day/1000 inhabitants). In Brasilia, cocaine and levamisole loads underwent an upsurge on Sunday, indicating a high consumption, and likely a direct disposal, of cocaine powder on this day. Conversely, the pyrolytic product resulting from the smoke of crack, anhydroecgonine methyl ester, and its metabolite anhydroecgonine were relatively stable over the four days, agreeing with a not recreational-associated use of crack.

Keywords: crack cocaine, sewage, solid-phase extraction, reversed-phase ion-exchange liquid chromatography, tandem mass spectrometry

1. Introduction

Estimating the consumption of illicit drugs by the analysis of wastewater (wastewater-based epidemiology, WBE) is a well-known approach currently applied in several countries worldwide[1-10]. This methodology is used to better understand the drug phenomenon at global level by institutions such as the European Monitoring Centre for Drugs and Drug Addiction[11], the Australian Criminal Intelligence Commission[12] or the New Zealand Police[13]. As one of the most consumed illicit substances, cocaine (COC) remains a major concern for governments and public agencies trying to monitor its trafficking routes, market expansion and prevalence of use. The increase of COC production over the last few years, driven by a dramatic resurgence of coca bush cultivation in Colombia[14], is now reflected in the increased availability, purity and use of the drug worldwide[11,14]. Thus, problems derived from high-risk patterns of consumption, including the smoking of the free-base form "crack", are expected to rise. Crack use, compared to snorting of COC hydrochloride, is associated to greater medical illness, higher mortality and psychiatric disorders and increased risk of polydrug use, among other negative consequences[15].

The urinary excretion profile of COC is well known and includes two major metabolites, ecgonine methyl ester (7-50% of a dose on molar basis) and benzoylecgonine (BE, 16-55%), which has been selected as the main biomarker of COC consumption in WBE studies[16-18]. In addition, the smoking of crack leads to the formation of a specific pyrolytic product, anhydroecgonine methyl ester (AEME or methylecgonidine), which is partially hydrolyzed in the human body to form anhydroecgonine (AE or ecgonidine)[16,19]. Both AEME and AE are excreted in urine and have been detected in urine samples from crack users[15,20] and in wastewater[19,21]. Their determination, together with BE, in the latter matrix allows to identify the different routes of administration of COC (co)existing in a specific location, as well as to follow potential changes in the patterns of abuse. Additionally, when COC is consumed

together with alcohol, a minor metabolite resulting from the transesterification of COC with ethanol is produced: cocaethylene (COE, 0.7% of a dose)[16]. This product has been used as a biomarker of the co-consumption of both substances by applying the WBE principles[22,23].

Most of the analytical methodologies developed for the determination of COC and its metabolites (mainly BE) in wastewater apply solid-phase extraction (SPE) followed by high performance or ultra-high performance liquid chromatography (HPLC or UHPLC) coupled to tandem mass spectrometry (MS/MS). Usually, separation is carried out on reversed-phase (RP) stationary phases modified with or containing embedded polar groups that provide a good separation efficiency in multi-residue analysis involving drugs of different chemical classes[21,24,25]. Hydrophilic interaction liquid chromatography (HILIC) has also been applied to improve the determination of small and very polar analytes that are poorly retained by traditional RPLC, such as ecgonine, ecgonine methyl ester, AE or AEME[19,26]. The recent commercialization of mixed-mode LC columns combining RP and ion-exchange properties may be a good alternative for the determination of very polar and ionizable compounds[27]. These columns contain long alkyl chain ligands with an ionizable terminus that, depending on the functional groups and the pH of the mobile phase, allow them to interact with the analytes by anion-exchange, cation-exchange or both, in addition to RP interactions. Several applications of this technology can be found in the pharmaceutical field[28-30] and, to a minor extent, in the food[31,32] and environmental field[33,34].

The aim of this study was to develop a new analytical method combining SPE and mixed-mode LC coupled to MS/MS for the determination of COC and its related compounds in wastewater. The analytes selected were the parent drug, its metabolites BE and COE, the pyrolytic products AE and AEME, and levamisole (LEV), one of the main pharmaceuticals used in the adulteration of COC hydrochloride[35]. To the best of our knowledge, this is the first application of RP ion-exchange mixed-mode LC for the determination of drugs of abuse. The optimized

and validated method was used to analyze composite 24-h raw wastewater samples collected in Santiago de Compostela (Spain) and Brasilia (Brazil) as a first proof of concept of its applicability in WBE. Brazil has experienced an upsurge in the abuse of crack in the last years[36] and, although still rare, there have been recent increases in the number of crack users in Italy and UK[11]. Therefore, the use of this method in the future in longer sampling campaigns could improve the understanding of the different patterns of COC consumption existing in different locations.

2. Materials and Methods

2.1. Chemicals and reagents

Analytical standards of COC, BE, COE and LEV were supplied by Cerilliant (Round Rock, TX, USA) as individual solutions of 1.0 mg/mL in methanol (MeOH). Isotopically labeled analogs (COC-D3, BE-D3, and COE-D3) were also supplied by Cerilliant as 0.1 mg/mL solutions in MeOH. AE hydrochloride (1.0 mg/mL in water) and AEME (1.0 mg/mL in acetonitrile (ACN)) were supplied by LGC Standards (Barcelona, Spain). Mixed stock solutions containing all the analytes or all the deuterated analogs (used as surrogate or internal standards, ISs) were prepared in MeOH and stored in the dark at -20 °C until use.

HPLC-grade MeOH, ACN, acetic acid (100%), hydrochloric acid (37%) and NH_3 solution in ultrapure water (25%) were supplied by Merck (Darmstadt, Germany). Formic acid (95-97%) and NH_3 solution in MeOH (7 N) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained in the laboratory by purifying demineralized water in a Milli-Q Gradient A-10 system (Merck-Millipore, Bedford, MA, USA).

2.2. Liquid chromatography-tandem mass spectrometry determination

Instrumental analyses were performed with a Waters Acquity UPLC® H class system (Milford, MA, USA) equipped with a quaternary solvent pump, a sample manager and a thermostated column compartment. Chromatographic separation was performed at 40 °C on an Acclaim® Mixed-Mode WCX-1 column (50×3.0 mm I.D., 3 μm particle size) from Thermo Scientific (Waltham, MA, USA). An Acclaim® Trinity P1 column (50×2.1 mm I.D., 3 μm particle size), also from Thermo Scientific and a Luna® Omega Polar C18 (50×2.1 mm I.D., 1.6 μm particle size) from Phenomenex (Torrance, CA, USA) were also used at the initial stage of the chromatographic optimization. In the final method, a dual eluent system consisting of (A) 10 mM of ammonium acetate in ultrapure water:ACN 90:10 at pH 3.5 and (B) 10 mM of ammonium acetate in ultrapure water:ACN 10:90 at an aqueous-equivalent pH of 3.5 was used at a flow rate of 0.2 mL/min. Gradient was as follows: 0 min (100% A), 2 min (100% A), 7 min (100% B), 10.5 min (100% B), 11 min (100% A), 18 min (100% A). Injection volume was set at 10 μL.

The UPLC® system was interfaced to a Xevo TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source. Nitrogen, provided by a nitrogen generator of Peak Scientific Spain (Barcelona, Spain), was used as desolvation gas at 650 L/h. Argon was employed as collision gas. The ESI interface operated in positive mode at a fixed capillary voltage of 3.4 kV and a temperature of 150 °C. Analytes were recorded in Selected Reaction Monitoring (SRM) mode acquiring one (IS), two (BE, LEV, COC, COE) or three (AE, AEME) precursor/product ion transitions per compound. For AE and AEME, three transitions were acquired to ensure their correct identification in the presence of interferences in real samples; two transitions and their ratio were the minimal criteria set to confirm their identity[37]. Transitions, optimal collision energy (CE) and cone voltage (CV) values were selected by direct infusion of individual standard solutions (10 μg/mL) in MeOH (Supplementary Material, SM, Table S1).

2.3. Sampling and sample treatment

Composite 24-h raw wastewater samples were collected at three different wastewater treatment plants (WWTP) located in Santiago de Compostela (NW of Spain) and Brasilia (the federal capital of Brazil). Sampling was performed during three (Santiago) or four (Brasilia) consecutive days in March (Santiago) and June (Brasilia) of 2018. In Brasilia, two WWTPs were considered: one serving the North of the city (Brasilia-North) and the other one receiving the wastewater of the southern neighborhoods (Brasilia South). Population served by each plant, sampling mode and daily flow rates are displayed in Table S2. Samples were solid-phase extracted daily following the optimized protocol developed in this study. Cartridges from Brasilia were frozen and shipped to our laboratory for analyte elution and instrumental analysis (performed within seven days after arrival). River water (for recovery experiments) was taken freshly from a creek in Santiago de Compostela.

Under final working conditions, sample aliquots (100 mL) were vacuum-filtered through 0.7 µm glass microfiber filters GF/A (Whatman, Kent, UK) and 0.45 µm cellulose filters (Merckmillipore, Bedford, MA, USA), acidified to pH 2.0 with hydrochloric acid 37% and spiked with 20 ng of ISs. SPE was performed onto mixed RP cation-exchange cartridges (Oasis MCX-150 mg, Waters) previously rinsed with 5 mL of MeOH and 5 mL of pH 2.0 ultrapure water. After sample percolation, sorbents were washed with 10 mL of pH 2.0 ultrapure water and dried under a nitrogen stream for ca. 30 min. Analytes were recovered with 2 mL of 2% NH_3 in MeOH. Eluates were evaporated to dryness under nitrogen and, immediately before injection, dissolved in 100 µL of LC mobile phase A (10 mM of ammonium acetate in ultrapure water:ACN 90:10 at pH 3.5) and filtered through 0.22 µm PVDF syringe-driven filters (Millex, Merckmillipore).

2.4. Method validation

BE, COC and COE were quantified using their corresponding deuterated analogs as IS; LEV using COC-D3 as IS; and AE and AEME by standard addition over the extract, since none of the three deuterated COC derivatives available in our laboratory corrected matrix effects for these two compounds.

Instrumental validation was performed in terms of linearity, intra- and inter-day precision, instrumental detection limits (IDLs) and instrumental quantification limits (IQLs). Calibration curves were prepared in mobile phase A (10 mM of ammonium acetate in ultrapure water:ACN 90:10 at pH 3.5) and ranged from IQLs to 10,000 ng/mL for BE, LEV, COC and COE, and from IQLs to 500 ng/mL for AE and AEME. ISs were added at 200 ng/mL. IDLs and IQLs were calculated as the concentration of a standard providing a signal-to-noise ratio (S/N) of 3 and 10, respectively. Precision was assessed at two concentration levels, 10 ng/mL and 100 ng/mL, by the relative standard deviation (%RSD) of six injections of a standard. Injections were performed over 24 h for the intra-day precision and over a period of several weeks for the inter-day precision.

The method validation parameters evaluated were method detection limits (MDL), method quantification limits (MQLs), matrix effects (ME), trueness and precision. MDLs and MQLs were calculated from IDLs and IQLs considering the concentration factor (1000) and ME. ME were expressed as the percentage of signal suppression (thus with a negative sign (-)) in a spiked SPE extract after non-spiked signal subtraction, referred to the signal of a standard of the same concentration.

Trueness and precision were assessed from recovery experiments in river water and raw wastewater performed in triplicate. River samples were spiked with 50 ng/L of all the analytes and 200 ng/L of IS, and wastewater samples with 500 ng/L of BE, LEV, COC and COE, 100 ng/L of AE and AEME and 200 ng/L of IS before extraction. Aliquots spiked only with IS

were processed simultaneously to account for the background levels of all the analytes in both matrices. These extracts were also used to prepare the standard addition calibration curves for the quantification of AE and AEME.

3. Results and Discussion

3.1. Chromatographic separation

Separation was initially attempted by UHPLC on a Luna® Omega Polar C18 column of 1.6 μm of particle size. This column contains a C18 stationary phase modified with polar groups that enhance the retention of polar compounds. A dual eluent system consisting of (A) ultrapure water and (B) MeOH was used at a flow rate of 0.5 mL/min. Different organic modifiers (formic acid 0.1%, ammonium acetate 5 mM at pH 3.5 or 5.5) were tried, but none of them affected either the peak shape or the analyte retention (data not shown). Alternatively, the gradient affected peak widening, with narrower peaks obtained when the elution started at 100% of A (gradients starting at 90%, 80% and 70% of A were also considered, analyzing, in every case, a standard prepared in the initial composition of the gradient). However, peak tailing was still observed for the analytes eluting earlier (LEV and, especially, AE and AEME (Fig. S1)) and, therefore, the use of mixed-mode RP ion-exchange HPLC was considered. Two columns were assessed: an Acclaim® Trinity P1 column that combines strong cation-exchange and weak anion-exchange properties (in addition to RP) and an Acclaim® Mixed-Mode WCX-1 column that incorporates weak cation-exchange functionalities. Supplier specifications, which do not recommend the use of MeOH as organic eluent and pH values outside the range 3-6.5, were taken into account to optimize the chromatographic separation. The eluent system consisted of: (A) ultrapure water with 10% of ACN, and (B) ACN with 10% of ultrapure water, both modified with 10 mM of ammonium acetate at pH 3.5 or at pH 5.5 (ultrapure water was added to B to guarantee the complete solubilization of the salt and, complementary, the same

percentage of ACN was added to A). Using a flow rate of 0.2 mL/min and an initial gradient composition of 100% of A, three pH combinations were assessed: both eluents at pH 3.5; both eluents at pH 5.5; A at pH 3.5 and B at pH 5.5. Fig. 1 shows the retention behaviour of the six analytes on (a) the WCX column and (b) the Trinity column. In the first case (Fig. 1 a), mainly RP interactions occurred at pH 3.5, providing a good retention for all analytes but the most polar ones (AE, BE and AEME), which eluted early in the chromatogram. When both eluents were adjusted to pH 5.5 and weak cationic exchange took place, retention of basic compounds (especially LEV and AEME) increased, whereas amphoteric species (AE and BE) were slightly less retained. In order to elute them the latest possible, mobile phase A was adjusted to pH 3.5. No differences were observed when combining it with mobile phase B adjusted to pH 3.5 or 5.5, so pH 3.5 was selected for eluent B and no gradient of pH was used with this column.

With the Trinity stationary phase (Fig. 1 b), the behaviour was different. At pH 3.5, analyte amine functionalities were protonated to a great extent and, therefore, all of them but BE were strongly retained by cationic exchange. At pH 5.5, cationic exchange decreased, and so did retention. Carboxylic moieties of amphoteric species (BE and AE) were partially deprotonated and repelled by the sulfonated nano-polymer beads of the outer surface of the column phase. This prevented them from interacting with the secondary amines (anion-exchange functionalities) in the inner pore area, causing a very early elution. The adjustment of mobile phase A to pH 3.5, maintaining mobile phase B at pH 5.5, avoided this effect, providing a good retention for all analytes. However, when wastewater extracts spiked with all the analytes were analyzed, the peak of AE splitted in the Trinity column, likely due to the presence of matrix components affecting its interaction with the stationary phase (SM, Fig. S2). This split was not observed with the WCX, which was, therefore, the column selected.

Both calibration standards and sample extracts were dissolved in mobile phase A (10 mM of ammonium acetate in ultrapure water:ACN 90:10 at pH 3.5) in accordance with the initial

gradient composition, and the injection volume was set at 10 µL. Fig. S3 of the SM shows the chromatogram for the first transition of all the analytes in a 100 ng/mL standard.

3.2. Sample preparation

Initial tests with 100 mL aliquots of spiked ultrapure water adjusted to pH 7.0 showed that AE was neither retained on Oasis HLB 200 mg cartridges nor on Oasis WCX 150 mg cartridges. Conversely, it was highly retained on Oasis MCX cartridges of 150 mg after passing samples adjusted to pH 2.0. Based on the demonstrated extraction efficiency of this sorbent for basic drugs and other amphoteric, more polar compounds[24,38,39], it was selected for the preconcentration of the analytes in this study. Sample pH, breakthrough volume and elution solvent volume were further assessed in detail.

The effect of the sample pH was investigated with 50 mL aliquots of ultrapure water spiked with 50 ng of all analytes and adjusted to pH 2, pH 4.5 and pH 7.0 (n=3). Samples were loaded under vacuum, cartridges dried under nitrogen for 30 min and analytes eluted with 10 mL of 5% NH_3 in MeOH. Eluates were evaporated to dryness and reconstituted in 100 µL of mobile phase A. For all the analytes but AE, no differences were observed at all assessed pHs (SM, Fig. S4). For AE, the response decreased with the increasing pH due to the higher prevalence of its deprotonated carboxylic form, in agreement with the extraction efficiency observed for this compound and also for AEME and ecgonine methyl ester in a previous study considering different sample pHs and Oasis MCX cartridges[19]. Therefore, samples were acidified to pH 2.0.

Experiments performed by passing 20 mL, 50 mL and 100 mL (n=3) of ultrapure water spiked with 50 ng of all analytes and adjusted to pH 2 through two cartridges connected in series demonstrated that the breakthrough was minimal even after passing 100 mL of sample (SM, Fig. S5). In this case, only 0.9% of AEME was recovered in the second cartridge; for the

remaining analytes, a percentage higher than 99.8% was recovered in the first cartridge. Thus, 100 mL was selected as sample volume. The sequential elution of MCX cartridges with four consecutive fractions of 2 mL of 2% NH_3 in MeOH showed that >92% of the recovered AE and >97% of the recovered BE, AEME, LEV, COC and COE were eluted in the first fraction (SM, Fig. S6). Therefore, 2 mL of 2% NH_3 in MeOH was selected as the elution solvent.

To avoid analyte degradation during sample transport and storage, samples were solid-phase extracted daily either in Santiago de Compostela or Brasilia, and cartridges from Brasilia were shipped frozen to our laboratory for instrumental analysis (performed within one week after arrival). Once submitted to SPE, analytes are stable for several weeks[40-42] and, therefore, no in-cartridge degradation is expected to occur under these conditions. In-sewer stability and stability during sampling were not specifically assessed in our study. However, previous works have shown that AE, AEME, BE and COE have a medium-to-high stability in wastewater[19,43,44] and, while COC is degraded in a remarkable extension to form BE[45, 46], the small percentage of excretion of COC in its parent form as compared to the excretion of BE limits the relevance of such transformation in WBE applications.

3.3. Method performance

Instrumental validation parameters (linearity, intra- and inter-day precision and instrumental detection and quantification limits) are displayed in Table 1. The linear range of the four analytes quantified by the use of ISs was IQL-10,000 ng/mL, with determination coefficients (R^2) varying between 0.9991 and 0.9999. For AE and AEME, external calibration responses were linear between their IQL and 500 ng/mL, with R^2 of 0.9905 and 0.9972, respectively. RSD values were below 8.0% and 29% for both a 10 ng/mL standard and a 100 ng/mL standard, for the intra-day and inter-day precision, respectively. IQLs were between 0.03 ng/mL for COC and 0.8 ng/mL for AEME.

Matrix effects in raw wastewater varied between -76% and -91% (spiking level: 500 ng/L of BE, LEV, COC and COE, 100 ng/L of AE and AEME). Recovery values in river water samples spiked with 50 ng/L of all the analytes varied between 96% and 121%, with %RSD between 2% and 21% (Table 1). In raw wastewater spiked with 500 ng/L of BE, LEV, COC and COE and with 100 ng/L of AE and AEME, they were between 93% and 113%, with %RSD between 0.3% and 18%.

Calculated MQLs ranged from 0.2 ng/L for COC and COE to 3 ng/L for AE and AEME. These values are in the same order of magnitude or lower (between 2 and 18 times lower for AEME, COC and COE) than the MQLs reported by Castiglioni et al.[19] and Baker and Kasprzyk-Hordern[24] following SPE-LC-MS/MS analyses. They are also considerably lower than the limits reached by Bisceglia et al. by a direct injection LC-MS/MS method[21], and by Sodré et al. following an SPE-LC-MS/MS protocol[36].

3.4. Concentrations and mass loads in 24-h composite wastewater samples

Table S3 shows the concentrations found in 24-h composite raw wastewater samples collected during three (Friday-Sunday, sampling from 9.00 to 9.00 of the following day) or four days (Thursday-Sunday, sampling from 00.00 to 00.00) in Santiago de Compostela and Brasilia, respectively. In Brasilia, the six analytes were quantified in all the samples collected at two different WWTPs: Brasilia North and Brasilia South. Fig. 2 shows the chromatogram (two transitions per analyte) of the sample collected in Brasilia North on Thursday. In Santiago de Compostela, AE and AEME were always below the MDL. BE was the substance found at the highest levels in all cases: COC was the second one, with exceptionally high levels in the samples of Sunday in Brasilia (over 200% with respect to the mean levels measured during the period Thursday-Saturday). This increase was accompanied by an upsurge in the levels of LEV (455% and 277% higher than the mean values of the other three days) suggesting a high

consumption (and likely a direct disposal) of COC hydrochloride. According to the Brazilian Federal Police, LEV is the main adulterant used in the salt form of COC, whereas another pharmaceutical, phenacetin, is mainly used to adulterate the free base[36]. COE and, when quantified, AE and AEME, were found at concentrations < 100 ng/L in all cases. To the best of author's knowledge, this is the second time that AEME is positively quantified in wastewater; only Bisceglia et al. had previously reported values of 15 ng/L in Baltimore[21].

Concentrations (ng/L) were multiplied by wastewater daily flow rates (L/day) to estimate mass excretion loads (mg/day), which were further normalized to the population served by each WWTP (Table 2). BE showed the highest population-normalized mass loads: up to 997 mg/day/1000 inhabitants in Brasilia North, 565 mg/day/1000 inhabitants in Brasilia South and 455 mg/day/1000 inhabitants in Santiago. In Brasilia, the increase in the concentrations of COC, LEV and COE in the samples of Sunday was also reflected in their mass loads. Conversely, mass loads of AE and AEME were relatively stable over the four days in Brasilia South or decreased slightly on Saturday and Sunday samples in Brasilia North (for AE). These findings agreed with the higher recreational use of snorted COC when compared to the more stable consumption of crack: the exclusive pyrolytic product and metabolite of the later remained relatively steady over the four days, whereas BE (common to COC hydrochloride and crack), LEV (main adulterant of COC powder) and COE (metabolite of the co-consumption of COC and ethanol) rose during the weekend.

A similar conclusion can be reached from the ratios between the concentrations of AE to BE (Fig. 3a): they decreased from 0.022 on Thursday to 0.009 (57% lower) on Sunday in Brasilia North and from 0.020 to 0.014 (34% lower) in Brasilia South. The ratio AEME/BE showed a great variation and did not allow to discern any clear trend (Fig. 3b). LEV/BE ratios (Fig. 3c) increased on Sunday in Brasilia, likely indicating a high consumption of adulterated COC powder on this day. It must be noted that the wastewater sampling in this city started at 00.00,

so the sample of Sunday includes the wastewater generated during Saturday-to-Sunday night. Co-consumption of alcohol and COC can be roughly estimated from COE/BE ratios, considering that the amount of COE excreted depends on the relative amounts of COC and ethanol ingested and that the amount of BE produced decreases in the presence of ethanol[16,47,48]. Based on the pharmacokinetic data available, Rodríguez-Álvarez et al. set a reference value of 0.039[23], so that lower COE/BE ratios indicate lower co-co-consumption of both substances. In this study, all the ratios reported were below this value. In Brasilia, a remarkable increase was observed on Sunday, whereas this increase happened on both Saturday and Sunday in Santiago (sampling here started at 9.00, so the wastewater generated during Saturday-to-Sunday night was mostly included in the sample of Saturday).

4. Conclusions

This study presents the first application of the RP-ion exchange mixed-mode liquid chromatography for the determination of drugs of abuse. The use of these stationary phases provides a good alternative to traditional RP and HILIC phases in the separation of very polar and ionizable compounds from other less polar species. COC, its metabolites and pyrolytic products and levamisole (used as adulterant) were successfully extracted from raw wastewater following an optimized SPE procedure and analyzed by HPLC-MS/MS with a RP-weak cation exchange chromatographic column. The application of the method to the analysis of composite samples of 24 h collected in Santiago de Compostela (Spain) and Brasilia (Brazil) provided the first proof of concept of its usefulness in WBE, allowing to get insights in COC patterns of consumption in terms of intake pathways, adulteration and co-consumption with alcohol.

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Conflict of interest: none

Supplementary Material (SM): Supplementary material is available online.

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Table 1. Performance parameters of the SPE-LC-MS/MS method: determination coefficients (R^2), relative standard deviations (%RSD), instrumental detection limits (IDL), instrumental quantification limits (IQL), matrix effects, percentages of recovery (%R), method detection limits (MDL) and method quantification limits (MQL).

Compound	IS	Linearity[a] (R^2)	Intra-day precision (%RSD, n=6)		Inter-day precision (%RSD, n=6)		IDL (ng/mL)	IQL (ng/mL)	Matrix effects (%Suppression and %RSD)	Trueness and precision (%R and %RSD)		MDL	MQL
			10 ng/mL	100 ng/mL	10 ng/mL	100 ng/mL			Wastewater[b]	River water[c]	Wastewater[b]	(ng/L)	(ng/L)
AE	-	0.9905	8.0	7.1	28	28	0.06	0.2	-91 (1)	121 (21)	111 (13)	0.7	2
BE	BE-D3	0.9999	2.7	2.0	8.1	5.6	0.1	0.4	-86 (1)	99 (5)	109 (2)	0.8	3
AEME	-	0.9972	4.7	3.5	18	14	0.2	0.8	-76 (8)	105 (2)	107 (18)	0.9	3
LEV	COC-D3	0.9991	2.7	1.2	20	14	0.03	0.1	-79 (4)	99 (4)	93 (6)	0.1	0.5
COC	COC-D3	0.9999	1.0	0.3	3.6	2.7	0.01	0.03	-79 (6)	96 (8)	113 (2)	0.05	0.2
COE	COE-D3	0.9999	0.7	1.2	2.3	4.6	0.01	0.04	-79 (6)	100 (6)	113.1 (0.3)	0.05	0.2

[a] Linear range: IQL-10,000 ng/mL for BE, LEV, COC and COE; IQL-500 ng/mL for AE and AEME. Level of BE, LEV, COC and COE internal standards (IS): 200 ng/mL
[b] Spiking level: 500 ng/L of BE, LEV, COC and COE, 100 ng/L of AE and AEME, 200 ng/L of IS
[c] Spiking level: 50 ng/L of all analytes, 200 ng/L of IS

Table 2. Population-normalized mass loads (mg/day/1000 inhabitants) for the substances quantified in 24-h composite raw wastewater samples collected during three or four days in March (Santiago) and June (Brasilia) of 2018. Days of the week: Thursday (Th), Friday (Fr), Saturday (Sa) and Sunday (Sun). NA: not applicable, due to concentration below the method detection limit.

Average load (mg/day/1000 inhabitants)	**AE**	**BE**	**AEME**	**LEV**	**COC**	**COE**
Santiago Fr	NA	314	NA	9.5	83	5.2
Santiago Sa	NA	455	NA	14	181	12
Santiago Sun	NA	420	NA	11	147	11
Brasilia North Th	14	655	6.9	26	84	1.4
Brasilia North Fr	16	848	6.8	43	142	3.1
Brasilia North Sa	14	997	12	59	152	4.3
Brasilia North Sun	8.5	920	6.4	159	255	17
Brasilia South Th	7.7	376	5.4	31	87	2.1
Brasilia South Fr	7.5	445	4.1	24	62	1.7
Brasilia South Sa	6.6	565	7.2	39	106	2.2
Brasilia South Sun	7.1	524	5.7	77	157	9

NA: not applicable due to concentration < MDL

Figure 1. Retention behaviour of the six analytes on (a) the WCX column and (b) the Trinity column using both mobile phases at pH 3.5 (red squares); both at pH 5.5 (blue circles); mobile phase (A) at pH 3.5 and (B) at pH 5.5 (orange triangle).

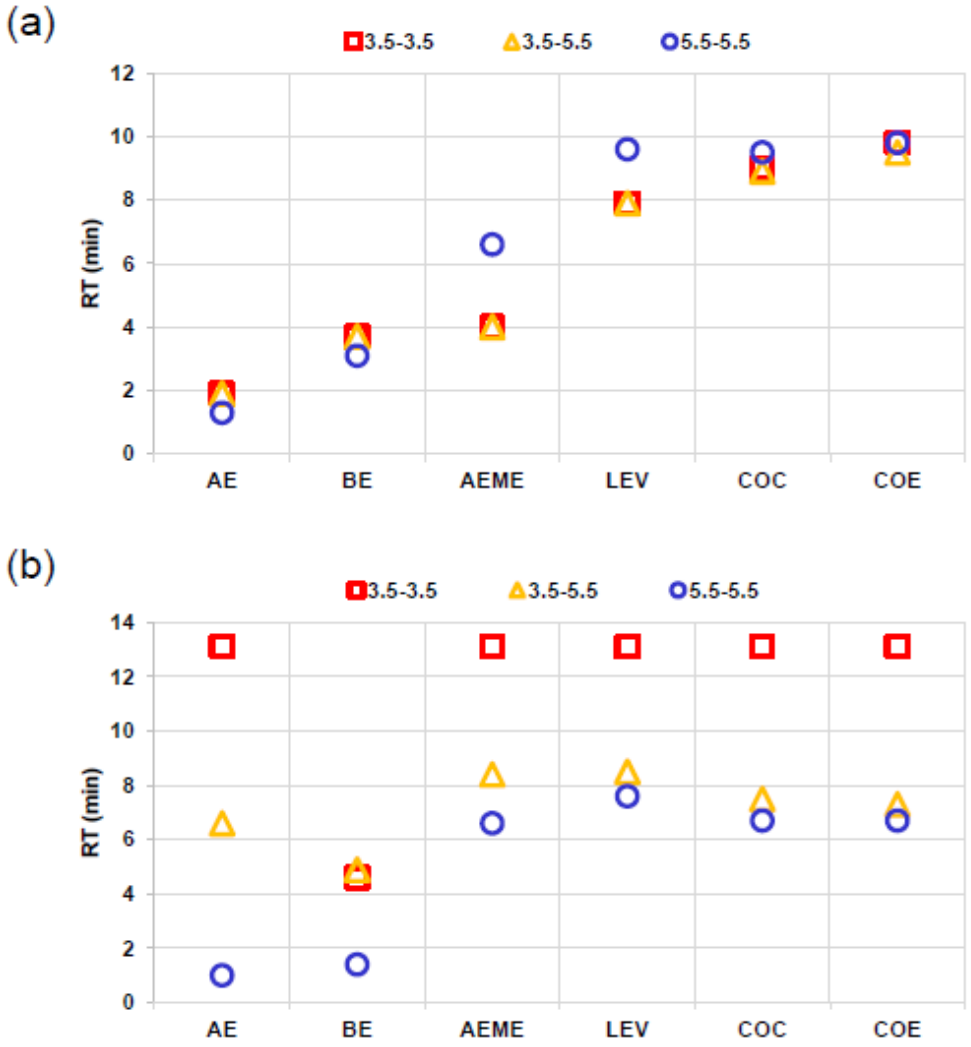


Figure 2. Chromatogram (two transitions per analyte) of the sample collected in Brasilia North on Thursday. In italics, ratio between transitions and percentage difference between this ratio (sample) and ratios in Table S1 (standard).

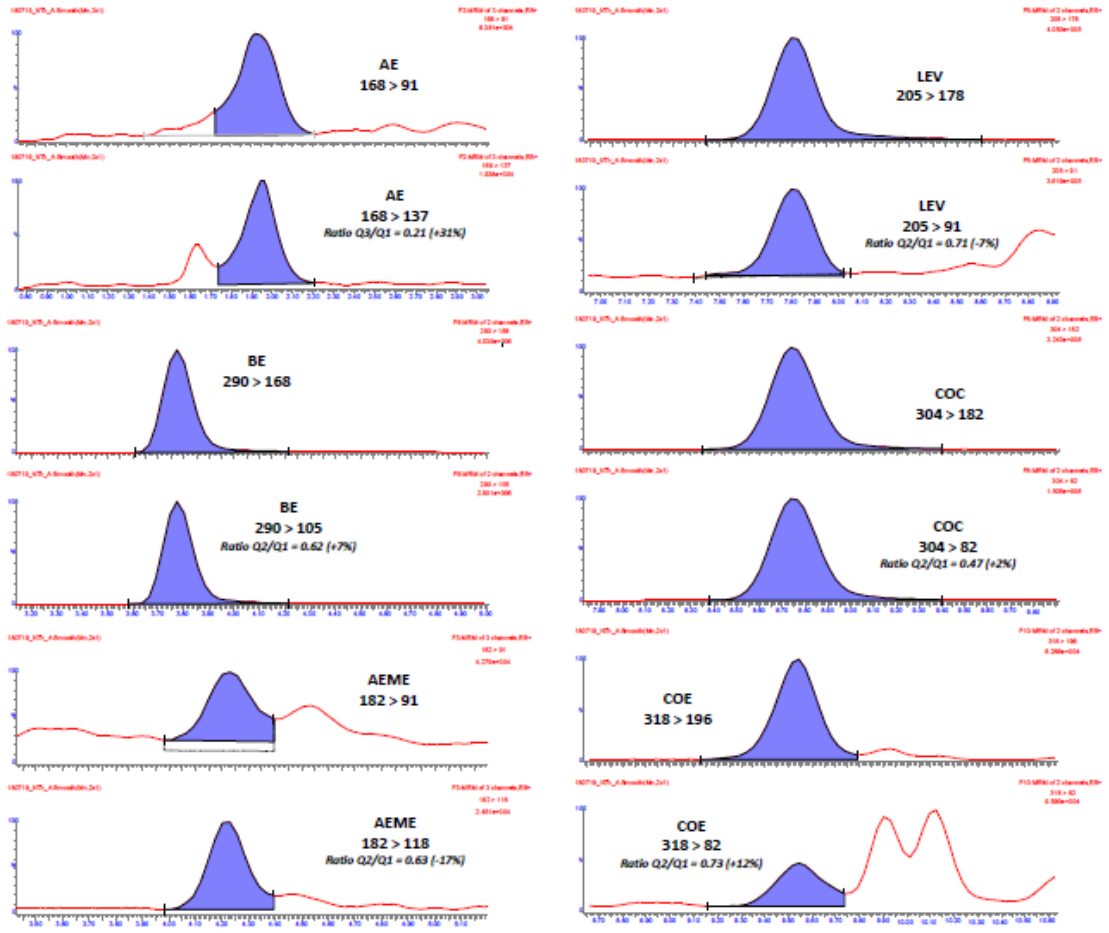
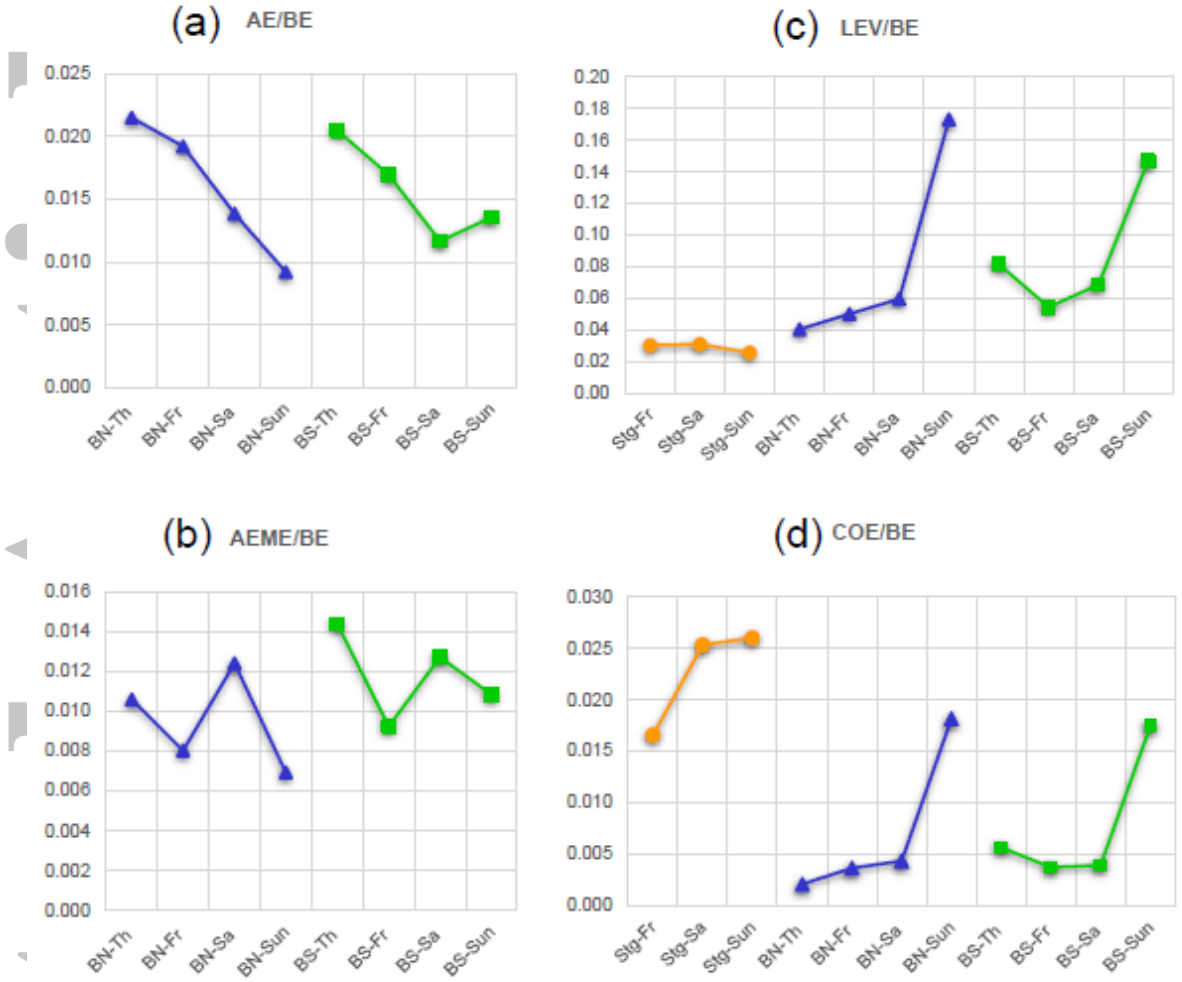


Figure 3. Ratios between the concentrations of different analytes and benzoylecgonine (BE) in Santiago de Compostela (Stg, orange circles), Brasilia North (BN, blue triangles) and Brasilia South (BS, green squares). Analytes: anhydroecgonine (AE), anhydroecgonine methyl ester (AEME), levamisole (LEV) and cocaethylene (COE). Days of the week: Thursday (Th), Friday (Fr), Saturday (Sa) and Sunday (Sun).



Drug Testing and Analysis

Supplementary Material to:

Profiling cocaine residues and pyrolytic products in wastewater by mixed-mode liquid chromatography-tandem mass spectrometry

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Table S1. Analytes, labeled compounds used as internal standards, retention time (RT) and SRM parameters: cone voltage (CV), transitions and collision energy (CE).

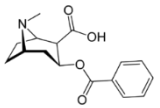
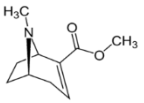
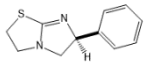
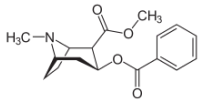
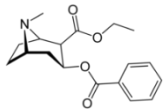
Compound	Structure	RT (min)	CV (V)	Q1	CE (V)	Q2	CE (V)	Q3	CE (V)	Ratio Q2/Q1	Ratio Q3/Q1
Anhydroecgonine AE		1.9	42	168>91	25	168>122	22	168>137	18	0.32	0.16
Benzoylecgonine BE		3.9	44	290>168	20	290>105	32	–	–	0.58	–
Anhydroecgonine methyl ester AEME		4.2	39	182>91	25	182>118	22	182>122	20	0.76	0.65
Levamisole LEV		7.9	48	205>178	22	205>91	36	–	–	0.76	–
Cocaine COC		8.9	30	304>182	22	304>82	32	–	–	0.46	–
Cocaethylene COE		9.7	32	318>196	22	318>82	32	–	–	0.65	–
BE-D3	–	3.9	44	293>171	20	–	–	–	–	–	–
COC-D3	–	8.9	30	307>185	22	–	–	–	–	–	–
COE-D3	–	9.7	32	321>199	22	–	–	–	–	–	–

Table S2. Characteristics of the wastewater treatment plants considered in this study: population served, sampling mode and daily flow rates. Weekdays: Th-Thursday, Fr-Friday, Sa-Saturday, Sun-Sunday.

WWTP	**Santiago de Compostela**	**Brasilia North**	**Brasilia South**
Population served	136,500	153,297	561,836
Method of estimation of the population	House connections × 2.5	Census (2017)	Census (2017)
Time of sampling beginning	9:00	00:00	00:00
Sampling mode	Time prop. (120 mL/10 min)	Flow prop.	Flow prop.
Flow (m^3/day) - Th	–	41,468	101,009
Flow (m^3/day) - Fr	110,231	45,191	100,328
Flow (m^3/day) - Sa	101,111	41,584	95,951
Flow (m^3/day) - Sun	97,817	34,985	87,635

Table S3. Analyte concentrations (mean in ng/L ± standard deviation) in composite samples of 24-h of raw wastewater collected in Santiago de Compostela and Brasilia during three or four days in March (Santiago) and June (Brasilia) of 2018. Days of the week: Thursday (Th), Friday (Fr), Saturday (Sa) and Sunday (Sun). <MDL: concentration below the method detection limit.

	Day	AE	BE	AEME	LEV	COC	COE
Santiago	Fr	<MDL	389±11	<MDL	12±3	102.7±0.1	6.4±0.7
	Sa	<MDL	614±23	<MDL	19±2	245±6	16±3
	Sun	<MDL	586±2	<MDL	15±1	205±5	15±3
Brasilia North	Th	52±2	2423±39	26±11	98±6	309±4	5.1±0.1
	Fr	55±5	2877±21	23±3	144±9	482±5	10.5±0.3
	Sa	51±12	3675±117	46±5	219±7	562±7	16.0±0.4
	Sun	37±1	4033±113	28±9	698±24	1117±31	73±2
Brasilia South	Th	43±6	2094±23	30±7	171±6	485±6	11.8±0.4
	Fr	42±2	2492±72	23±1	135±2	349±3	9.3±0.1
	Sa	39±2	3305±51	42±6	227±3	623±11	13.1±0.3
	Sun	46±7	3360±68	36±5	493±9	1008±10	59±1

Figure S1. Chromatogram (first transition of all analytes) of a 100 ng/mL standard analyzed with the Luna® Omega Polar C18 column. Mobile phases: (A) 0.1% formic acid in ultrapure water, (B) 0.1% formic acid in MeOH. Gradient: 0 min (100% A), 0.5 min (100% A), 2.5 min (100% B), 4.5 min (100% B), 4.51 min (100% A), 6 min (100% A). Flow rate 0.5 mL/min.

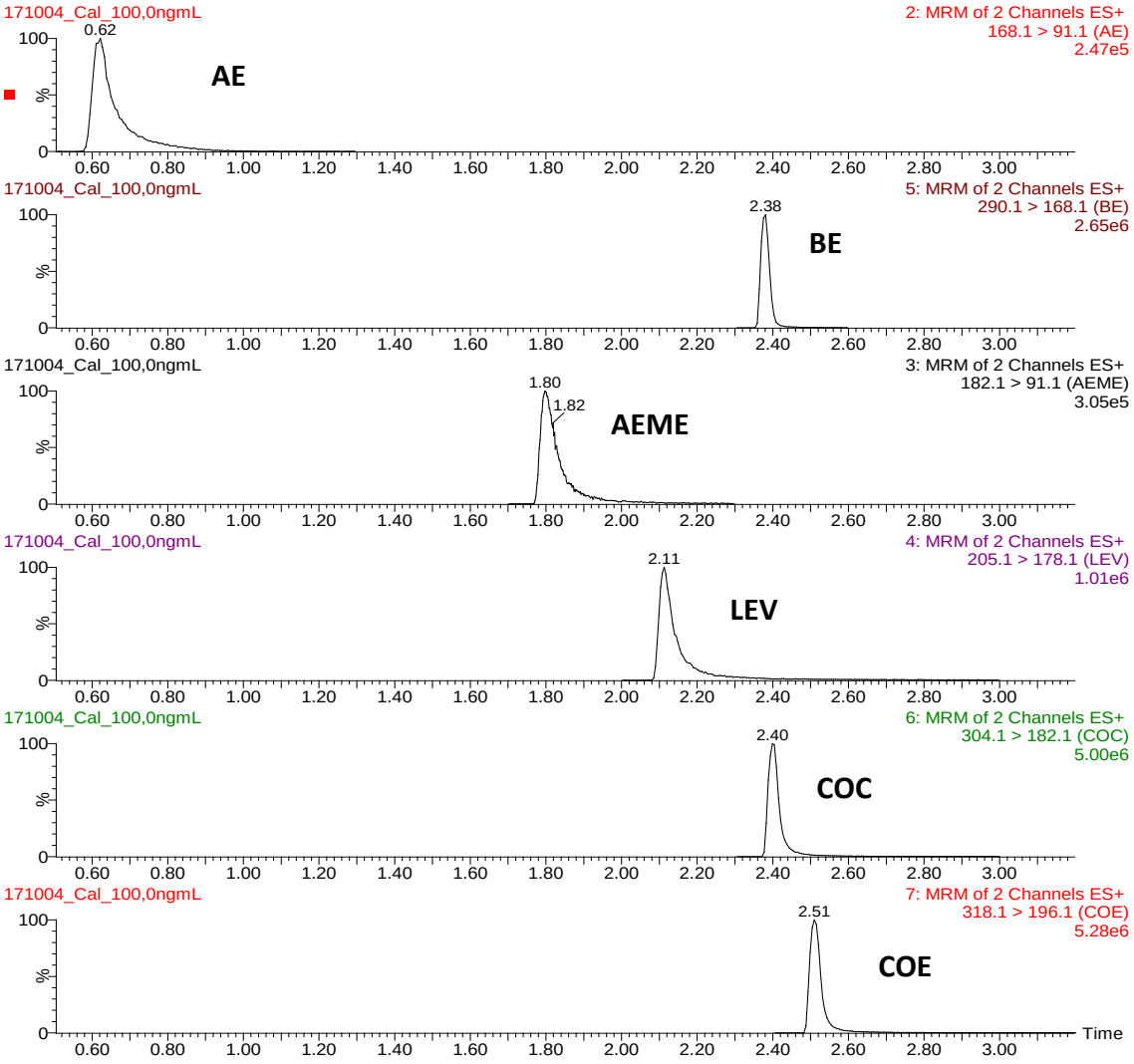


Figure S2. Chromatogram for the first transition of anhydroecgonine (AE) in an SPE wastewater extract spiked with 500 ng/mL of AE and analyzed with (a) the Acclaim® Trinity P1 column; and (b) the Acclaim® Mixed-Mode WCX-1 column.

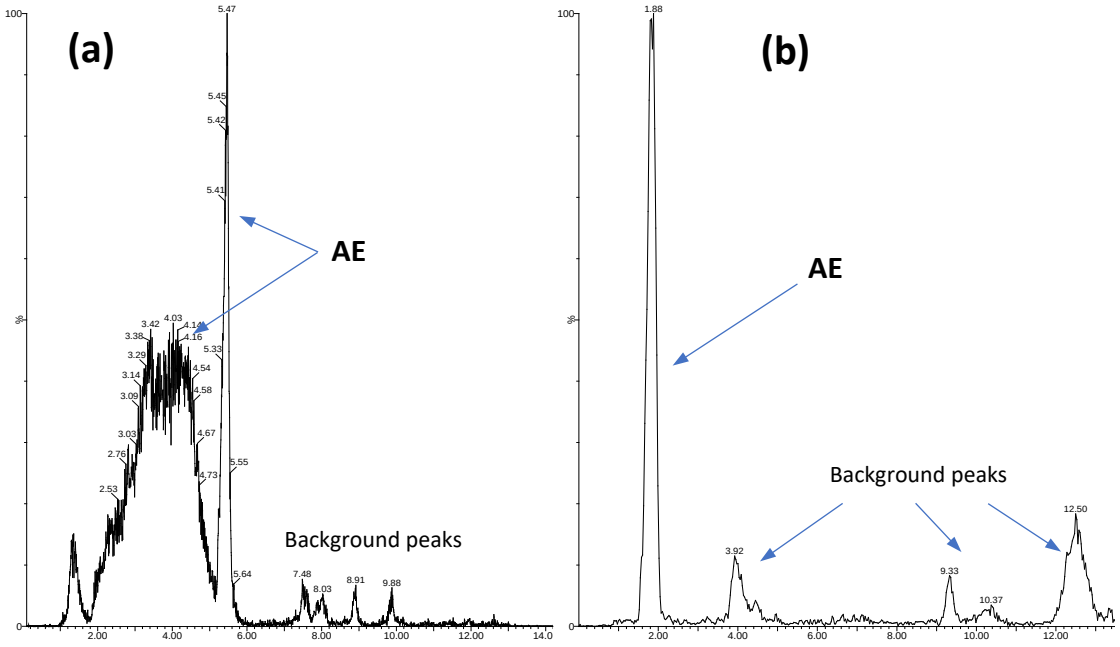
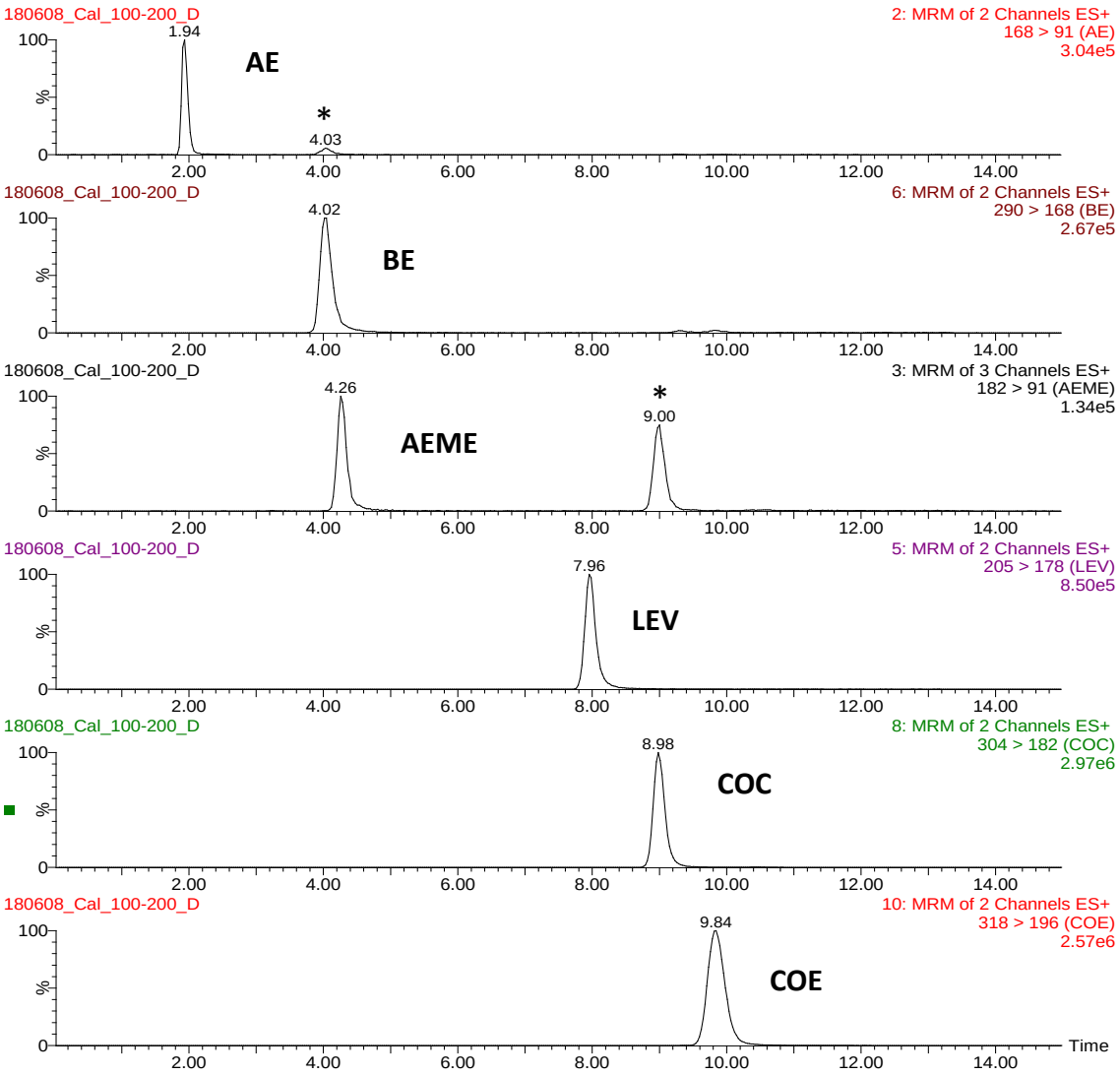


Figure S3. Chromatogram (first transition of all analytes) of a 100 ng/mL standard under final working conditions with the Acclaim® Mixed-Mode WCX-1 column.



* Artefact peaks from BE and COC in-source fragmentation

Figure S4. Influence of sample pH on analyte relative recovery during SPE.

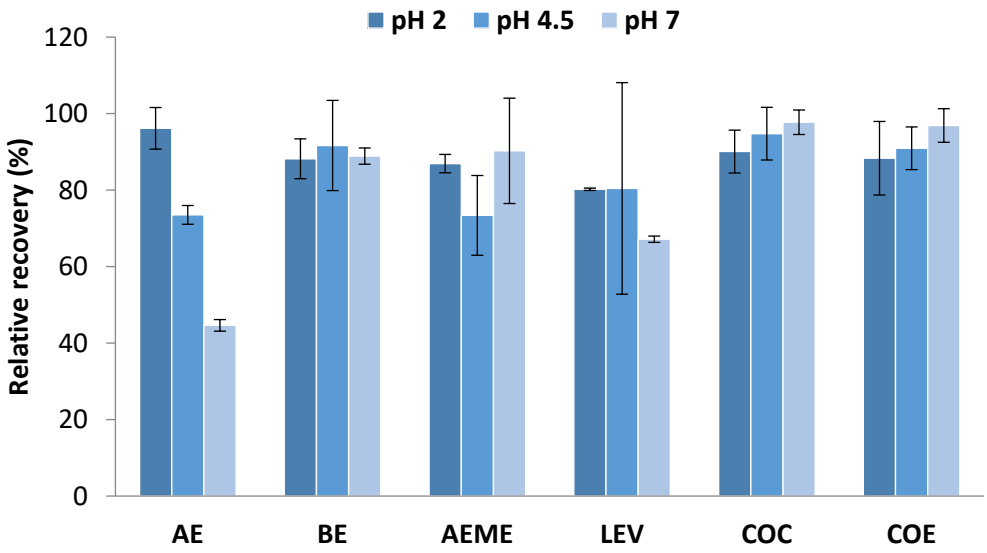


Figure S5. Analyte relative recovery on the first and second cartridge (connected in series) after passing 100 mL of ultrapure water at pH 2 spiked with 50 ng of all analytes.

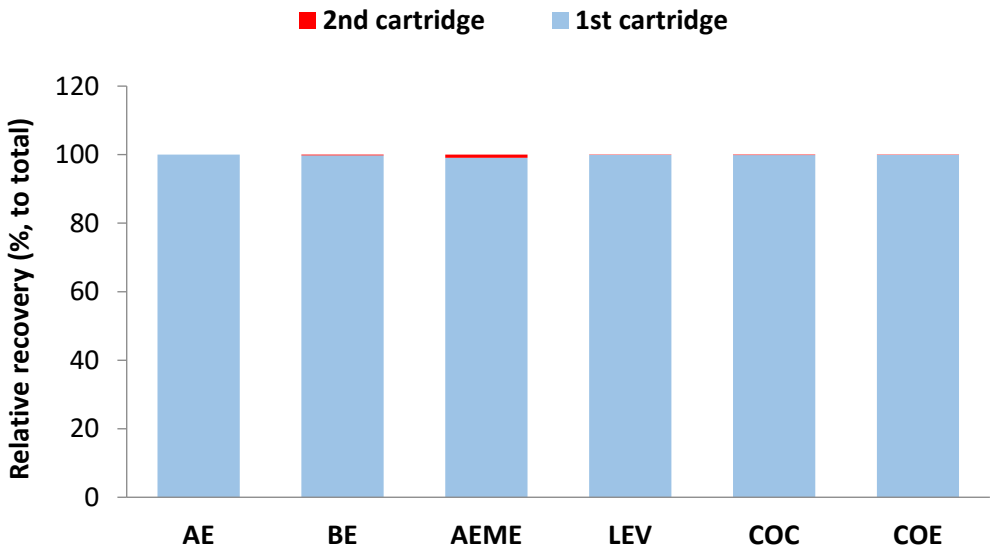


Figure S6. Analyte relative recovery on successive elution fractions of 2 mL of 2% NH_3 in MeOH.