Anal Bioanal Chem (2010) 398:2701–2712
DOI 10.1007/s00216-010-4191-9

ORIGINAL PAPER

Quantification of drugs of abuse in municipal wastewater via SPE and direct injection liquid chromatography mass spectrometry

Kevin J. Bisceglia · A. Lynn Roberts · Michele M. Schantz · Katrice A. Lippa

Received: 2 June 2010 / Revised: 30 August 2010 / Accepted: 2 September 2010 / Published online: 24 September 2010
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Abstract We present an isotopic-dilution direct injection reversed-phase liquid chromatography–tandem mass spectrometry method for the simultaneous determination of 23 drugs of abuse, drug metabolites, and human-use markers in municipal wastewater. The method places particular emphasis on cocaine; it includes 11 of its metabolites to facilitate assessment of routes of administration and to enhance the accuracy of estimates of cocaine consumption. Four opioids (6-acetylmorphine, morphine, hydrocodone, and oxycodone) are also included, along with five phenylamine drugs (amphetamine, methamphetamine, 3,4-methylenedioxy-methamphetamine, methylbenzodioxolyl-butanamine, and 3,4-methylenedioxy-*N*-ethylamphetamine) and two human-use markers (cotinine and creatinine). The method is sufficiently sensitive to directly quantify (without preconcentration) 18 analytes in wastewater at concentrations less than 50 ng/L. We also present a modified version of this method that incorporates solid-phase extraction to further enhance sensitivity. The method includes a confirmatory LC separation (selected by evaluating 13 unique chromatographic phases) that has been evaluated using National Institute of Standards and Technology Standard Reference Material 1511 Multi-Drugs of Abuse in Freeze-Dried Urine. Seven analytes (ecgonine methyl ester, ecgonine ethyl ester, anhydroecgonine methyl ester, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoyl-ecgonine, ecgonine, and anhydroecgonine) were detected for the first time in a wastewater sample.

Keywords Wastewater · Illicit drugs · Urinary metabolites · LC/MS/MS · Direct injection · SPE

Electronic supplementary material The online version of this article (doi:10.1007/s00216-010-4191-9) contains supplementary material, which is available to authorized users.

K. J. Bisceglia · A. L. Roberts
Department of Geography and Environmental Engineering,
Johns Hopkins University,
3400 North Charles Street,
Baltimore, MD 21218, USA

K. J. Bisceglia · M. M. Schantz · K. A. Lippa (✉)
Analytical Chemistry Division,
National Institute of Standards and Technology,
100 Bureau Drive, Stop 8392,
Gaithersburg, MD 20899-8392, USA
e-mail: katrice.lippa@nist.gov

Present Address:
K. J. Bisceglia
Chemistry Department, Bard High School Early College,
30-20 Thomson Avenue,
Queens, NY 11101, USA

Introduction

The analysis of drugs of abuse in wastewater (increasingly called sewer epidemiology) has emerged as an appealing means for routinely monitoring drug consumption in municipalities. The approach is unobtrusive and has the potential to provide data at much better temporal and spatial resolution than is afforded by traditional interview-based survey sampling. Since first being proposed [1], "drug tests" have been conducted on a number of regions [2–15]. Such studies, which rely almost exclusively on reversed-phase liquid chromatography–tandem mass spectrometry (RPLC/MS/MS), have successfully measured the parent and/or metabolites of many drugs of abuse, including cocaine, heroin, prescription pain killers, amphetamine and methamphetamine, ecstasy (3,4-methylenedioxy-methamphetamine or MDMA), and marijuana [11].

Cocaine is particularly well suited to sewer epidemiology. It is excreted almost exclusively (85% to 90%) in urine [16–

18], predominantly as the parent drug and three metabolites: benzoylecgonine, ecgonine methyl ester, and ecgonine [19]. Its human metabolites are all chemically unique in that they have no licit sources. Equally important, the urinary profile of cocaine metabolites is dependent on the route of administration (ROA); metabolites can include pyrolysis products such as anhydroecgonine and anhydroecgonine methyl ester if smoked as crack cocaine [20, 21], or ethylated derivatives such as cocaethylene and ecgonine ethyl ester if co-ingested with alcohol [21, 22]. These minor metabolites can provide valuable information on lifestyle behaviors associated with cocaine use; their inclusion in wastewater monitoring programs could provide data useful to public health and law enforcement communities. At present, although cocaine has been the most extensively investigated illicit drug in municipal wastewater, such analyses have been limited to the parent drug, two of its three major metabolites (benzoylecgonine and ecgonine methyl ester), and three minor metabolites, only one of which (cocaethylene) can provide ROA information [11, 14, 15].

Expanding cocaine monitoring programs to include additional metabolites has another benefit in that, by capturing a greater fraction of the total cocaine load, it might be possible to reduce uncertainties in wastewater-derived measurements of cocaine consumption. Current procedures, which entail a number of assumptions [6, 14, 23–26], estimate cocaine usage from a single metabolite (usually benzoylecgonine), multiplying measured concentrations by a correction factor that incorporates the molar mass ratio (parent/metabolite) as well as the fraction excreted as the metabolite in question [6, 14, 23–26]. Using a total measured cocaine load rather than a single metabolite would reduce errors that might be associated with individual variations in the extent of metabolism, or with the degradation of key metabolites within the sewage system [27]. Ecgonine, in particular, can represent as much as 23% of total cocaine in human urine [19]; as the final hydrolysis product of cocaine, it is likely to represent an even greater fraction of the total cocaine load in wastewater. To our knowledge, there are no published LC/MS/MS methods for the analysis of ecgonine in municipal sewage, although an initial reversed-phase LC/MS/MS methodology by Peck et al. that would capture a broad array of cocaine metabolites (including ecgonine) has been previously reported [28].

The small molecular size, high polarity, zwitterionic character, and structural similarity of most cocaine metabolites make them challenging analytes in matrices as complex as municipal wastewater. The low molecular mass metabolites, such as ecgonine and anhydroecgonine, are seldom investigated, even in drug screening tests or forensic studies. When such metabolites are analyzed, gas chromatography–mass spectrometry (after derivatization) has been the technique of choice [29, 30], although it is insufficiently sensitive to detect most cocaine metabolites in environmental samples. RPLC/MS/MS has been employed with some success [28, 31, 32], although the poor retention of these analytes makes them susceptible to matrix effects and interferences from salts [33]. Recently, Gheorghe et al. [10] and Giroud et al. [34] used hydrophilic interaction chromatography (HILIC) to successfully quantify the alkyl ester metabolites of cocaine (ecgonine methyl ester and ethyl ester, and anhydroecgonine methyl ester) in body fluids and tissue [34] and in wastewater [10]. Gheorghe et al. [10] also reported the successful separation of ecgonine using HILIC (though this metabolite was not detected in wastewater samples and solid-phase extraction (SPE) recoveries were not reported). The ability of HILIC/MS/MS to successfully quantify complex mixtures of illicit drug metabolites remains, however, untested.

Most current methods for the analysis of drugs of abuse in wastewater incorporate a sample pre-concentration step. Off-line SPE is the most commonly used approach [11], although online SPE [12] has also been employed with notable success. These techniques can be costly and time consuming, and sometimes require specialized instrumentation. In addition, they can concentrate interferences along with target analytes. While such approaches have historically been necessary for the analysis of illicit drugs and pharmaceuticals in environmental samples, the additional cost and labor involved have limited the spatial and temporal resolution at which analyses could be performed. Fortunately, recent improvements in MS/MS sensitivity may enable the analysis of at least some drugs of abuse via direct injection. This is especially likely for the major cocaine metabolites, which are routinely measured in the 100 to 1,000 ng/L range [11]. In a notable recent advancement, Chiaia et al. [9] used large volume injection and RPLC/MS/MS to quantify 22 chemicals (with an emphasis on illicit drugs) in municipal wastewater. Unfortunately, their analyte suite included only three cocaine metabolites along with the parent drug.

Sewer epidemiology holds great promise as a tool for monitoring the population-based consumption of drugs of abuse. At present, however, this approach is still in its infancy. A need remains for robust and reliable methods that can estimate drug use with minimal analytical effort and reduced measurement uncertainty. In this paper and with our recent hydrolysis method for the simplified analysis of total cocaine residues [35], we provide improved methods for determining cocaine residues in municipal wastewater using LC/MS/MS. The RPLC method described herein maximizes the information that is obtained from each measurement by simultaneously analyzing cocaine and 11 of its metabolites, including those with the potential to provide ROA information. In addition to cocaine, this method includes four

opioids (6-acetylmorphine, morphine, hydrocodone, and oxycodone), five phenylamine drugs (amphetamine, methamphetamine, MDMA, methylbenzodioxolyl-butanamine (MBDB), and 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA)), and two human-use markers (cotinine, a nicotine metabolite, and creatinine, a human urine marker), bringing the total number of analytes to 23 (see Fig. S1 in the Electronic Supplementary Material for molecular structures and CAS numbers). Because this method successfully quantifies more than 99% (by mass fraction) of the cocaine that is potentially present in human urine [19], it drastically reduces the uncertainty involved in extrapolating levels of usage from occurrence measurements [27]. Moreover, because this method is sensitive enough to analyze 18 analytes via direct injection, it reduces both the time and cost of routine monitoring, thus streamlining analysis. We also optimized a confirmatory separation that provides an independent verification of wastewater measurements. Confirmatory analyses are routinely employed in regulatory measurements and will likely be an important component of any official program to monitor drugs of abuse in wastewater.

Materials and methods

Chemicals Details on the procurement, storage, and handling of analyte and isotopically labeled surrogate standards, as well as on the preparation of spiking and calibration solutions, are presented in the Electronic Supplementary Material. All chemicals used in this study were of reagent quality or better.

LC separations We evaluated analyte retention on 13 different LC columns that included eight columns containing embedded polar groups (EPGs) and three HILIC or normal-phase materials, one of which (Ultra IBD) can also be operated in both reversed-phase and HILIC modes. These are summarized in Table S1 in the Electronic Supplementary Material. LC phases were tested under different pH, temperature, solvent, and gradient conditions to fully assess their suitability for this study. At a minimum, each column was evaluated at both 35 and 55 °C, and at pH 2.9 and 5.9 (buffered with 10 mmol/L ammonium formate and 10 mmol/L ammonium acetate, respectively), using acetonitrile as the organic modifier. In addition, six columns (Ascentis RP-Amide, Atlantis T3, Gemini NX, Polaris RP-Amide, Polaris C18-Ether, and Viva PFPP) were further tested with each combination of pH and temperature using methanol as the modifier, and Gemini NX was tested at an additional pH of 10.3 (using 10 mmol/L ammonium bicarbonate as buffer).

If the retention of early-eluting analytes was deemed acceptable relative to the column void volume (i.e., $k' \geq 0.4$) under any of these conditions, then the gradient volume was systematically varied to optimize analyte resolution and peak shape. The LC phase and conditions that yielded the best combination of analyte retention, resolution, and peak shape were chosen for the primary separation. A second, confirmatory separation was selected to maximize the difference in elution order from the primary separation.

Quality assurance/quality control (QA/QC) and wastewater samples were analyzed using the following optimized chromatographic procedures: the primary separation was achieved on a Restek Viva PFPP column, using a guard column of the same stationary phase (2.1×10 mm, 5 μm). The separation occurred at 55 °C in a water/acetonitrile mobile phase, both containing 0.1% formic acid. The proportion of organic solvent increased from 5% to 25% in 20 min, from 25% to 95% in 2.5 min, and was held at 95% for 2.5 min. The column was rinsed and re-equilibrated by holding at 50% acetonitrile for 10 min, and then at 5% acetonitrile for 25 min. The second, confirmatory separation was effected on the Restek Ultra IBD column, operating in reversed phase at 55 °C. An IBD guard column (2.1×10 mm, 3 μm) was used. The separation occurred in 10 mmol/L ammonium acetate (pH 5.9)/acetonitrile by ramping from 3% to 20% acetonitrile in 34 min, from 20% to 60% in 2 min, and then holding at 95% for 4 min. The column was re-equilibrated at 3% acetonitrile for 30 min. Both separations used an Agilent 1,200 series Binary LC system with vacuum degasser and autosampler, at a flow rate of 200 μL/min. A 0.2 μm in-line frit (Upchurch Scientific, Oak Harbor, WA, USA) was installed between the autosampler and the LC column. An injection volume of 5 μL was used in all analyses.

Solid-phase extraction Analyte recoveries were investigated on six different SPE sorbent media (Oasis HLB, Oasis MCX, Strata X, Strata XC, Bond Elut Certify, and CleanScreen DAU), according to manufacturers' guidelines for the extraction of basic analytes. Details are provided in the Electronic Supplementary Material. In the final SPE procedure, Strata XC cartridges (500 mg; 12 mL) were conditioned under gravity with 10 mL each of methanol and deionized water (adjusted to pH 2 using 1 mol/L HCl). Next, they were loaded under vacuum with 200 mL of sample, also adjusted to pH 2, at a rate of 6 mL/min. After loading, the cartridges were washed under gravity with 10 mL of pH 2 water, 10 mL of 2% formic acid, and 10 mL of 5% methanol (aqueous). Post-wash, the cartridges were dried at 34 kPa for 20 min, were sealed in plastic, and were stored overnight at −20 °C. After reaching room temperature, cartridges were eluted under gravity by 10 mL of 2% ammonium hydroxide in methanol, freshly prepared. Eluent volume was reduced to 500 μL in a TurboVap at 35 °C, under a constant stream of N_2 (g). In the last step, each 500 μL aliquot was transferred to an LC sample vial and was diluted with 1 mL of 0.1% formic acid. All samples were then analyzed using the LC separations described above.

MS/MS parameter optimization Positive electrospray ionization MS/MS analysis was performed using a triple quadrupole mass spectrometer (API 5000) equipped with a QJet ion guide and a Turbo V Ionspray source (Applied Biosystems Inc, Foster City, CA, USA). Declustering potentials and collision energies were optimized separately for each analyte via direct infusion of 100 μg/L solutions; results are listed in Table 1. Entrance and collision cell exit potentials were set at 10 and 15 V for all analytes. Ion source parameters were optimized under LC flow conditions (200 μL/min) using a 50 μg/L solution of all analytes. All quantitative analyses were performed using a source temperature of 600 °C, an ion spray voltage of 5,500, and curtain, nebulizer, and turbo gas pressures of 345, 207, and 276 kPa, respectively. Signal intensity for the primary ion transitions (precursor/product I in Table 1) was used as the sole optimization criterion for all parameters.

Analyte quantitation and QA/QC Quantification was performed by scheduled multiple-reaction monitoring (MRM), using an MRM detection window of 180 s for the primary separation and 240 s for the confirmatory separation. A target scan time of 13 s was used for both separations. Analytes were quantified by isotopic dilution, using the labeled surrogates listed in Table 1. Each analyte (with the exception of creatinine) was monitored with two ion transitions. The most abundant transition (precursor/product I in Table 1) was used for quantification, while the second transition (precursor/product II) was used for verification. Labeled surrogates and creatinine were monitored and quantified using only the most abundant ion transitions.

Limits of detection (LODs) were determined by the direct injection of calibrant solutions ranging in concentration from 10 ng/L to 25 μg/L. The calibrants were prepared in a substitute wastewater that had been created according to ASTM protocol D5905-98 [35]. Briefly, the substitute

Table 1 Abbreviations, surrogate identities, and analyte-specific RPLC/MS/MS method parameters for the target analytes

Target analyte	Abbreviation	Surrogate[a]	Precursor ion (*m*/*z*)	Product ion I (*m*/*z*)	Product ion II (*m*/*z*)	DP (eV)	CE (eV)	Retention time (min)	
								PFPP[b]	IBD-RP[c]
Ecgonine	EC	EC-d_3	186.1	168.3	150.1	65	35	2.72	3.13
Creatinine	CR	AEC-d_3	114.0	44.0	NA	12	26	2.85	2.93
Ecgonine methyl ester	EME	EME-d_3	200.1	182.1	150.1	45	35	3.13	5.71
Anhydroecgonine	AEC	AEC-d_3	168.1	91.0	122.3	85	38	3.32	3.06
Cotinine	CT	CT-d_3	177.1	117.9	146.2	40	45	3.47	13.61
Ecgonine ethyl ester	EEE	EME-d_3	214.1	196.2	150.1	40	40	3.82	7.20
Morphine	MO	MO-d_6	286.1	165.1	181.1	75	50	5.42	12.40
Anhydroecgonine methyl ester	AEME	AEME-d_3	182.1	118.1	122.3	50	37	5.89	10.23
Amphetamine	AM	AM-d_5	136.1	91.3	119.3	21	20	11.20	12.27
p-Hydroxybenzoylecgonine	pOHBE	pOHBE-d_3	306.1	168.2	186.4	60	32	11.97	15.72
m-Hydroxybenzoylecgonine	mOHBE	pOHBE-d_3	306.1	168.2	186.4	60	32	12.91	16.60
Methamphetamine	MA	MA-d_5	150.1	91.3	119.3	40	22	13.37	17.73
Oxycodone	OC	OC-d_6	316.1	241.2	256.1	65	33	13.94	20.12
6-Acetylmorphine	AcMO	AcMO-d_3	328.1	165.2	181.1	55	50	14.08	24.92
Hydrocodone	HC	HC-d_6	300.1	199.1	213.2	75	45	15.12	31.06
3,4-Methylenedioxy-methamphetamine	MDMA	MDMA-d_5	194.1	163.1	133.1	40	22	15.27	19.61
Benzoylecgonine	BE	BE-d_3	290.1	168.2	150.1	40	30	15.88	21.90
Norbenzoylecgonine	NBE	BE-d_3	276.2	136.1	154.0	80	30	17.59	17.51
3,4-Methylenedioxy-*N*-ethylamphetamine	MDEA	MBDB-d_5	208.1	163.1	133.1	35	23	17.78	23.54
Methylbenzodioxolyl-butanamine	MBDB	MBDB-d_5	208.1	135.1	177.2	45	22	19.40	26.35
Cocaine	COC	COC-d_3	304.1	182.1	150.1	60	27	25.23	37.32
Norcocaine	NC	COC-d_3	290.1	136.0	168.1	65	28	25.91	39.06
Cocaethylene	CE	CE-d_3	318.1	196.1	150.1	60	30	27.60	42.01

DP declustering potential, *CE* collision energy

[a] The same DP and CE values are used for labeled (surrogate) and unlabeled (target) analytes

[b] Restek pentafluorophenyl-propyl column

[c] Restek IBD Ultra column operated under reversed-phase conditions

wastewater is designed to be a reproducible simulation of municipal sewage, and is intended for use in method evaluation. It consists primarily of water, salt, clay, surfactant, and beer. LODs for direct injection were computed from the calibration curve using the method of Hubaux and Vos [37], allowing a 5% probability of false positives. LODs for the method with SPE were computed from the direct injection LODs, using a concentration factor of 133.3 and adjusting for analyte recoveries.

Method reproducibility was evaluated by determining intra-day accuracy and precision for the analysis of triplicate standards. Standards analyzed by direct injection contained 1 μg/L of each analyte in 0.1% formic acid. The full SPE, LC/MS/MS method was evaluated with deionized water samples that had been spiked to the following levels: 125 ng/L for 6-acetylmorphine, cocaethylene, ecgonine ethyl ester, norcocaine, MBDB, MDEA, MDMA, anhydroecgonine methyl ester, *p*-hydoxybenzoylecgonine, and *m*-hydroxybenzoylecgonine; 380 ng/L for anhydroecgonine; 400 ng/L for norbenzoylecgonine, ecgonine methyl ester, oxycodone, hydrocodone, methamphetamine, and amphetamine; 2,630 ng/L for cocaine, cotinine, and morphine; 7,620 ng/L for ecgonine; 8,025 ng/L for benzoylecgonine; 502 μg/L for creatinine. Accuracy and precision were also evaluated through the triplicate analysis of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1511 Multi-Drugs of Abuse in Freeze-Dried Urine, which contains 162±8 μg/L of benzoylecgonine and 309±20 μg/L of morphine in human urine [38]. Samples analyzed by direct injection were diluted 1:100 with 0.1% formic acid, and samples analyzed with the full SPE, LC/MS/MS method were diluted 1:500 in pH 2 deionized water. Method repeatability was investigated by performing multiple injections of analyte standards. The magnitude of ionization enhancement/suppression in untreated wastewater was evaluated relative to deionized water for both direct injection and the full SPE, LC/MS/MS method, using 50 μg/L of analytes in each matrix. All QA/QC determinations described above were performed with the primary LC separation.

Application to wastewater A 24-h, flow-weighted composite sample (4 L) of wastewater influent (post-screening) was collected from the Back River Wastewater Treatment Plant (BRWWTP) in Baltimore, MD, USA on April 30, 2009. The BRWWTP serves approximately 944,000 people in the greater Baltimore area. The plant flow on the day of sample collection was 405×10^6 L/day. The sample was transported to our laboratory on dry ice, whereupon it was split into subsamples. One 1,200-mL subsample was prepared for analysis by the full SPE, RPLC/MS/MS method. The subsample was amended with isotopically labeled surrogates, was filtered through 1.2 μm Millipore GF/C filters (Bedford, MA, USA) to remove suspended solids, and was acidified to pH 2 with 1 mol/L HCl. A 100 mL subsample for direct injection analysis was also amended with labeled surrogates and was filtered through 1.2 μm GF/C filters, whereupon it was filtered through 0.22-μm nylon filters (Whatman, Kent, ME, USA) and adjusted to 0.07% formic acid (volume fraction). The processed subsamples were stored at −20 °C for 48 h and were allowed to reach room temperature before SPE (extracting 200 mL aliquots, n=5) or direct injection (5 mL for each injection, n=8), followed by RPLC/MS/MS analysis. SPE extracts (n=5) were also analyzed using the confirmatory (reversed-phase IBD) separation.

Results and discussion

LC separations A multiple-ion chromatogram for a standard solution of target analytes on the Restek Viva PFPP column (primary separation column) is provided in Fig. 1a along with MRM chromatograms of the dominant transitions (precursor/product I) for the cocaine analytes in Fig. S2 in the Electronic Supplementary Material. Corresponding retention times for each analyte are included in Table 1. Retention factors (k', computed using a column void volume of 0.326 mL) for ecgonine and anhydroecgonine were 0.41 and 0.91, respectively. Analytes elute in three broad groupings: (1) the small, highly polar, and weakly aromatic analytes (e.g., ecgonine, anhydroecgonine and their alkyl esters, creatinine, and cotinine); (2) the phenylamine drugs, opioid drugs, and benzoylecgonine (and substituted benzoylecgonine) cocaine metabolites; and (3) cocaine and its close structural analogs, norcocaine and cocaethylene. Chromatographic resolution proved sufficient to enable the trace-level analysis of all 23 analytes using two MRM transitions. Multiple-reaction monitoring chromatograms are presented for all analytes in Fig. S2 of the Electronic Supplementary Material. Peak asymmetry is minimal for all analytes except hydrocodone and oxycodone, which exhibit slight peak fronting. Interestingly, no peak fronting was observed for 6-acetylmorphine and morphine, the other opioid drugs.

Confirmatory separations were performed using a Restek Ultra IBD column in reversed-phase mode at 55 °C and pH 5.9 (10 mmol/L ammonium acetate/acetonitrile). Multiple ion chromatograms obtained for the two columns are compared in Fig. 1a, b, and retention times are included in Table 1. Retention factors for ecgonine and anhydroecgonine (k', void volume=0.326 mL) were 0.92 and 0.87, respectively. Chromatographic resolution is slightly improved over the primary separation, although peak tailing is more pronounced. Peak shape for anhydroecgonine methyl ester was too poor for accurate quantitation. Most impor-

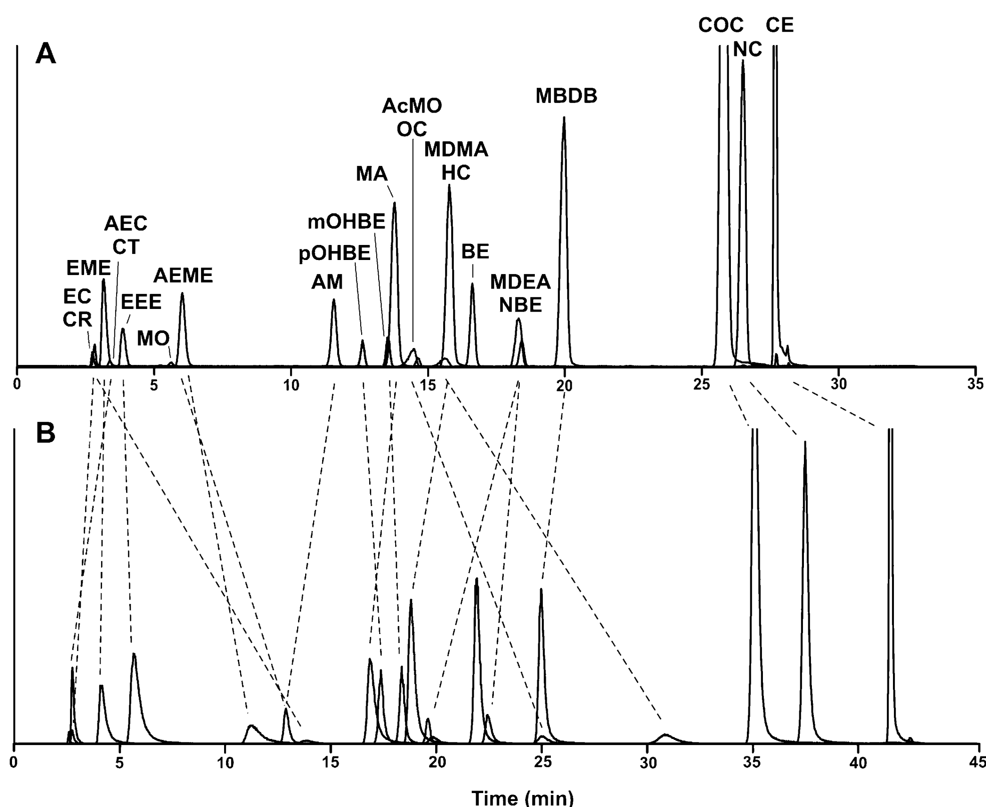

Fig. 1 A comparison of selectivity between the primary (pentafluorophenyl-propyl; **a**) and confirmatory (IBD; **b**) RPLC separations in the analysis for 80 μg/L of target analytes in deionized water. Refer to Table 1 for analyte abbreviations

tant, there are a total of 17 retention time shifts between the primary and confirmatory separations (Fig. 1a, b). All analytes that co-elute in the primary separation are separated, including anhydroecgonine and cotinine, MDEA and norbenzoylecgonine, MDMA and hydrocodone, 6-acetylmorphine and morphine, and cocaine and norcocaine.

Both the pentafluorophenyl-propyl (PFPP) and IBD phases exhibit normal-phase characteristics, which can result in U-shaped retention for some analytes [39]. This phenomenon is demonstrated for cocaine, norcocaine, and cocaethylene on the PFPP phase in Fig. S3. Such behavior is essential to the performance of these columns, but it also necessitates relatively long re-equilibration periods between runs. We also found that rinsing the PFPP column between runs with a water/acetonitrile (50/50 by volume) mobile phase containing 0.1% formic acid helped to reduce background interferences.

After the chemical functionality of the bonded phase, mobile phase composition is the most powerful variable influencing analyte retention and selectivity (i.e., elution order) in LC separations. In developing the LC separations, particular attention was paid to the mobile phase pH, as all analytes in the study possess ionizable functional groups. Changing the mobile phase pH from 2.9 to 5.9 generally increased retention (and altered selectivity) for the cocaine metabolites possessing carboxylic acid moieties, but did so at the expense of peak shape. Retention of the other analytes—which contain protonated nitrogen atoms below pH 8—did not change appreciably with pH, even at pH 10.3. The identity of the mobile phase organic modifier did not have a substantial influence on the retention of early-eluting analytes, or on selectivity, though analyte resolution was slightly better in acetonitrile than in methanol for most columns. Column temperature has also been reported to influence analyte selectivity, particularly when the pH of the mobile phase is close to the pK_a(s) of one or more analytes [40]. For the columns we investigated, increasing the temperature from 35 to 55 °C produced only minimal changes in analyte selectivity. This was true at pH 2.9 and pH 5.9, even though the carboxylic acid moieties of the cocaine metabolites have pK_a values between 3.0 and 3.5.

With one exception [10], all published LC/MS/MS methods for the detection of drugs of abuse in wastewater have employed C_{18} phases to effect chromatographic separations. Though each of the three C_{18} phases investigated in this study sufficiently resolved most analytes when operated with acetonitrile or methanol at pH 2.9, they could not adequately retain ecgonine and anhydroecgonine under any of the conditions studied. EPG phases have been specifically developed to retain highly polar analytes, and may provide a useful (though largely untested) alternative to conventional C_{18} phases. We evaluated eight EPG phases, spanning a range of chemistries. In general, peak tailing was slightly more pronounced than with the C_{18} phases but was usually acceptable. The amide EPG phases

(Ascentis RP-Amide and Polaris RP Amide) provided better analyte resolution than the ether (Polaris C_{18}-Ether) and propyl cyano (Allure Basix) phases under the conditions tested. The pentafluorophenyl (PFP) and PFPP phases provided the best combination of resolution and retention, and were investigated in detail. Peak shape and retention for all analytes was strongly influenced by the presence of the propyl spacer and the silica pore size. By increasing the distance between the PFP and the silica surface, the propyl spacer may reduce electrostatic repulsion between the protonated analytes and the protonated silica surface, thus enhancing analyte retention. The influence of silica pore size on retention is more curious, given the small molecular radii of our analytes. Nonetheless, best results were obtained with 300 Å silica.

HILIC is receiving increased attention for its ability to successfully retain small, highly polar metabolites [41, 42]. It has been successfully used to analyze cocaine, benzoylecgonine, and ecgonine methyl ester in municipal wastewater [10, 15]. We investigated three HILIC phases, each with different chemistries. The elution order of many analytes in HILIC mode was inverted relative to that observed under reversed-phase conditions, making HILIC an attractive option for developing confirmatory separations. Retention of all analytes, and especially ecgonine and anhydroecgonine, was also dramatically improved over the RPLC separations. Unfortunately, analyte resolution was exceedingly poor under all pH, temperature, solvent, and gradient conditions considered and could not even be improved by operating isocratically. Chromatograms demonstrating the effect of solvent gradient on analyte resolution under HILIC conditions are presented in Fig. S4 of the Electronic Supplementary Material. While it seems that HILIC can be a valuable alternative to RP chromatography in many cases, it may not be well suited for separation of large numbers of structurally similar analytes.

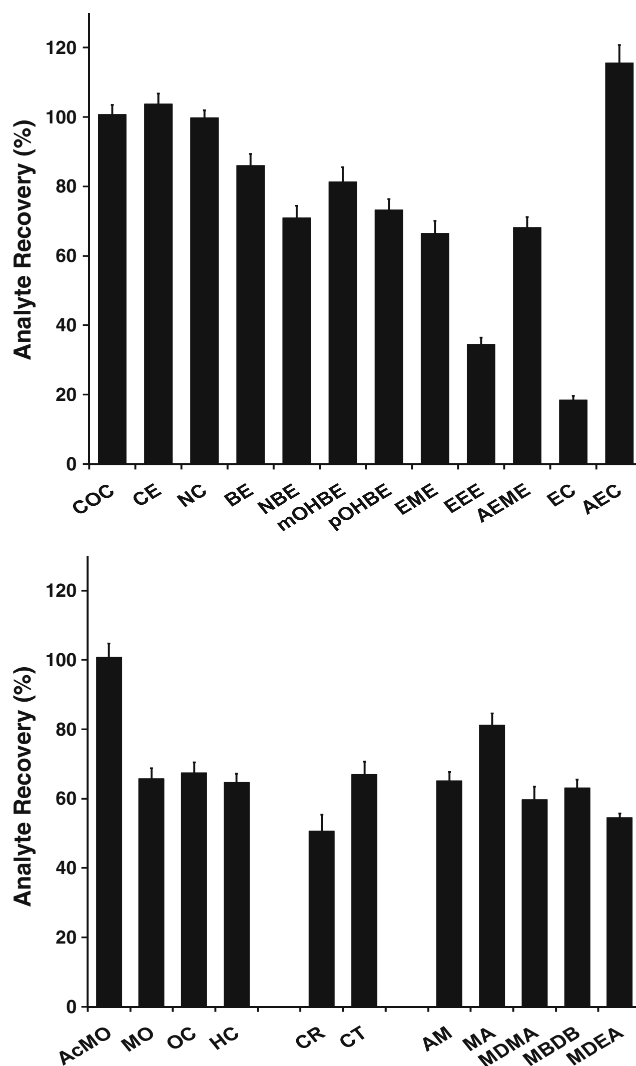

Fig. 2 SPE recoveries (using Strata XC) for 200 ng/L of target analyte in deionized water. *Error bars* represent 95% confidence intervals on seven replicates. Refer to Table 1 for analyte abbreviations

Solid-phase extraction The six mixed-mode and cation exchange SPE sorbents investigated were chosen for screening because most have been successfully employed to pre-concentrate basic drugs of abuse from municipal wastewater [11]. We believe, however, that this is the first time that CleanScreen DAU has been considered for the analysis of environmental samples. Each cartridge was used according to its manufacturer's protocol for the extraction of basic drugs; no individual optimization was performed. Under the conditions tested, Strata XC provided the best recoveries for the majority of the analytes. Recoveries obtained in deionized water using the final SPE method are presented in Fig. 2. Recovery values are slightly higher than those reported by others using Strata XC [6], and are slightly lower than reported for Oasis MCX [3, 8], a closely related sorbent. They are within the range of values reported for Oasis HLB [11], the most commonly used sorbent.

In general, recoveries for the cocaine analytes appear to be dependent on their level of esterification. Complete recovery was observed for cocaine, cocaethylene, and norcocaine, while recoveries for the benzoyl ester-containing metabolites ranged from 71% to 86%. Recoveries for the methyl esters of ecgonine and anhydroecgonine were slightly lower, at 67%, and the recovery of ecgonine was lower still (19%). This pattern may be expected, as π–π interactions between the benzoyl esters and the divinylbenzene backbone of Strata XC are likely to promote sorption, while electrostatic repulsion between hydrolyzed carboxylates and surface-bound sulfonates is likely to inhibit sorption. Recoveries of ecgonine ethyl ester (35%) and anhydroecgonine (115%) are very notable

Table 2 Limits of detection (LODs) and precision and accuracy data for the analysis of the target analytes using the primary separation

Target analyte	LOD (ng/L)		Distilled water (n=3)				Human urine (NIST SRM 1511; n=3)			
			With SPE[a]		Direct injection[b]		With SPE[c]		Direct injection[d]	
	Direct injection[e]	With SPE[f]	Precision (% RSD)	Accuracy (% of Target)	Precision (% RSD)	Accuracy (% of Target)	Precision (% RSD)	Accuracy (% of Target)	Precision (% RSD)	Accuracy (% of Target)
Creatinine	8,130	120	14.1	118	–[g]	–[g]				
Ecgonine	800	33	1.62	102	4.84	112				
Ecgonine methyl ester	32	0.36	1.28	102	4.24	96				
Anhydroecgonine	2,500	19	2.97	101	–[g]	–[g]				
Cotinine	47	0.53	2.59	100	16.5	108				
Ecgonine ethyl ester	179	3.9	3.90	100	6.64	99				
Morphine	670	7.7	1.11	101	5.08	97	1.88	102	3.52	103
Anhydroecgonine methyl ester	35	0.38	0.47	125	1.20	92				
Amphetamine	37	0.43	2.09	102	7.95	103				
p-Hydroxybenzoylecgonine	18	0.18	2.79	102	2.67	110				
m-Hydroxybenzoylecgonine	16	0.15	3.45	103	4.62	107				
Methamphetamine	15	0.14	17.8	99	3.19	103				
Oxycodone	10	0.11	0.42	101	3.65	102				
6-Acetylmorphine	22	0.16	1.53	102	3.96	103				
Hydrocodone	16	0.19	4.53	103	3.73	105				
MDMA	15	0.19	14.0	187	0.91	99				
Benzoylecgonine	33	0.29	2.11	102	1.33	105	0.85	100	2.64	104
Norbenzoylecgonine	35	0.37	1.76	102	3.84	104				
MDEA	4	0.05	>90	112	10.3	114				
MBDB	4	0.05	24.5	116	0.78	100				
Cocaine	4	0.03	1.98	101	0.32	110				
Norcocaine	3	0.02	4.87	102	2.30	97				
Cocaethylene	4	0.03	3.44	100	2.33	113				

[a] Spiking levels: 125 ng/L for AcMO, CE, EEE, NC, MBDB, MDEA, MDMA, AEME, pOHBE, and mOHBE; 400 ng/L for NBE, EME, OC, HC, MA, and AM; 2,630 ng/L for COC, CT, and MO; 502 µg/L for CR; 8,025 ng/L for BE; 380 ng/L for AEC; 7,620 ng/L for EC

[b] Spiking levels: 1,000 ng/L for all analytes

[c] Spiking levels: 627 ng/L for MO and 330 ng/L for BE

[d] Spiking levels: 3,180 ng/L for MO and 1,680 ng/L for BE

[e] LODs determined for direct injection RPLC(PFPP)/MS/MS in substitute wastewater using the method of Hubaux and Vos [38]

[f] LODs for SPE, LC/MS/MS were estimated from direct injection LODs, using an SPE concentration factor of 133.3 and adjusting for analyte recovery

[g] Evaluated concentration (1,000 ng/L) is below the LOD

exceptions to this trend, however, and highlight the importance of having isotopically labeled surrogates for each analyte under investigation. Recoveries for the opioid drugs, phenylamine drugs, and human-use markers were mostly around 60%, though disparate values for 6-acetylmorphine and morphine, and amphetamine and methamphetamine, provide further evidence that small changes in structure can substantially influence recovery.

Quantification via direct injection The determination of LODs in real wastewater is often complicated by the background occurrence of target analytes. The most commonly employed alternative is to perform such determinations in purified water, which can result in unrealistic lower values. Substitute wastewater [36] offers a complex yet reproducible matrix for measuring LODs, one without interferences from analyte contamination. Despite its obvious benefits, we believe that our study is the first to utilize substitute wastewater to determine LODs for trace-level organic contaminants. These LODs are listed in Table 2. They were statistically determined from a calibration curve of directly injected standards, allowing a 5% probability of false positives (i.e., the LOD is the lowest concentration having a 95% probability of being larger than background) [37]. LODs were also computed from the same data using a signal-to-noise ratio of three and were found to be within a factor of two of values reported in Table 2.

For analysis via direct injection RPLC/MS/MS, 18 out of 23 analytes have LODs less than 50 ng/L in substitute wastewater, and 11 have LODs less than 20 ng/L. LODs are larger for early-eluting analytes. This is most notable for ecgonine, creatinine, anhydroecgonine, and morphine, for which LODs (in ng/L) are between 650 and 8,200 ng/L. The high levels of occurrence of creatinine [9] and morphine [4] in wastewater influent may, nonetheless, enable their analysis via direct injection, bringing the total number of analytes that can potentially be quantified without preconcentration to 20. Only anhydroecgonine, ecgonine, and ecgonine ethyl ester do not appear amenable to analysis in wastewater via direct injection. LODs for analysis via SPE were on average two orders of magnitude lower than for direct injection and reflect preconcentration and individual analyte recovery (Table 2).

Intra-day accuracy and precision were evaluated in deionized water, both for analysis via the full SPE, RPLC/MS/MS method and via direct injection (Table 2). Precision errors for the full method (including SPE) are generally less

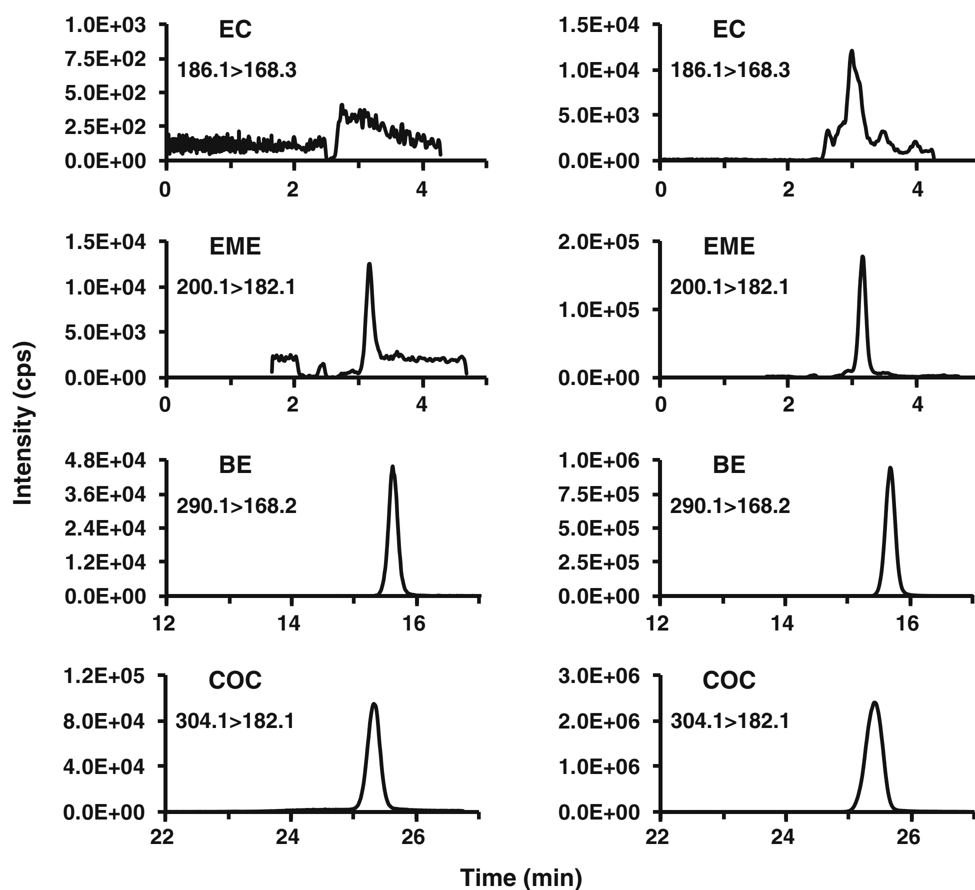

Fig. 3 Multiple-reaction monitoring chromatograms for precursor-to-product I transitions of ecgonine (*EC*), ecgonine methyl ester (*EME*), benzoylecgonine (*BE*), and cocaine (*COC*) in an unamended wastewater sample analyzed via direct injection LC/MS/MS (*left*) and via SPE, LC/MS/MS (*right*). Refer to Table 3 for analyte concentrations

than 5%, and are less than 2% for eight of the 23 analytes. The accuracy determination reveals a slight over-prediction bias, though errors are also within 5% for most analytes. Both the accuracy and precision data indicate problems in the analysis of creatinine and the phenylamine drugs (except for amphetamine) using the full SPE, RPLC/MS/MS method. Analyses conducted during method development displayed similarly large errors for creatinine, which may result from its early elution, or from the lack of an isotopically labeled internal standard. Reported errors for the phenylamine drugs are much larger than those we obtained in preliminary investigations, however. They are also in disagreement with errors reported during SPE recovery (Fig. 3) and the analysis of wastewater samples using the full SPE, LC/MS/MS method [35].

Accuracy and precision for analysis by direct injection RPLC/MS/MS were evaluated at relatively low concentrations (1,000 ng/L) closer to the analyte LODs. Even so, errors for both metrics are less than 10% for all analytes but MDEA and cotinine. Unfortunately, this low concentration was below the LODs for creatinine and anhydroecgonine (8,200 and 2,500 ng/L, respectively); we did not, therefore, evaluate accuracy and precision for these compounds. Notably, the phenylamine drugs, with the partial exception of MDEA, were better quantified by direct injection RPLC/MS/MS than by the full SPE method.

Calibration curves for all analytes are linear over the tested calibrant range (10 ng/L to 25 μg/L), though cocaine, norcocaine, and cocaethylene exhibit nonlinearity above 75 μg/L. Calibration correlation coefficients were greater than 0.99 for all analytes. Independent investigations of accuracy and precision were also obtained by analyzing the two NIST-certified drugs of abuse components, benzoylecgonine and morphine, in freeze-dried human urine [38]. Accuracy and precision values for human urine (Table 2) are comparable with those obtained in deionized water for both direct injection and the full SPE method.

A comprehensive assessment of matrix effects on an LC-MS/MS method's precision and accuracy may involve the determination of relative effects on samples of various

Table 3 Average concentrations (±1 standard deviation) in ng/L of target analytes in Back River (BRWWTP) influent, as determined using different preparative and chromatographic techniques

Target analyte	Solid-phase extraction (SPE)		Direct injection[a]
	Primary PFPP[b]	Secondary IBD RP[c]	Primary PFPP
Cocaine and metabolites			
Cocaine	806±15	800±34	788±11
Benzoylecgonine	2,690±50	2,660±113	2,790±32
Ecgonine methyl ester	412±4.5	430±9.6	417±28
Ecgonine	1,150±39	1,060±92	<LOD
Norcocaine	981±0.4	23±0.7	22±1.6
Norbenzoylecgonine	195±24	144±29	237±22
p-Hydroxybenzoylecgonine	54±1.3	54±1.7	67±4.6
m-Hydroxybenzoylecgonine	18±2.4	16±2.1	43±5.1
Cocaethylene	18±1.4	18±0.5	24±0.8
Ecgonine ethyl ester	33±1.6	36±1.4	291±27
Anhydroecgonine methyl ester	15±0.5	NA	<LOD
Anhydroecgonine	92±2.9	72±2.3	<LOD
Phenylamine drugs			
Amphetamine	45±1.0	45±2.7	<LOD
Methamphetamine	225±1.6	280±8.9	194±13
MDMA	22±2.3	20±1.3	16±1.4
MBDB	ND	ND	ND
MDEA	ND	ND	ND
Opioid drugs			
6-Acetylmorphine	23±1.1	21±0.6	<LOD
Morphine	997±1.2	1,000±33	970±69
Oxycodone	288±7.2	300±8.3	315±24
Hydrocodone	59±0.9	51±1.6	68±6.0
Human-use markers			
Creatinine	311±70×10^3	137±8×10^3	121±4×10^3
Cotinine	2,050±84	2,170±84	1,970±297

<LOD less than the limit of detection, *NA* not analyzed, *ND* not detected

[a] SPE, RPLC/MS/MS analysis using the primary (PFPP) separation (*n*=5)

[b] SPE, RPLC/MS/MS analysis using the confirmatory (IBD RP) separation (*n*=5)

[c] Direct injection RPLC/MS/MS analysis (without SPE) using the primary (PFPP) separation (*n*=8)

sources. In some cases, it may be necessary to eliminate such effects from a particular step within the assay [43]. Towards such an assessment, the relative effect of the wastewater matrix was evaluated for our methodology by comparing peak areas obtained for 50 μg/L of analyte in wastewater via direct injection analysis and in SPE extracts of wastewater (after subtracting background concentrations), relative to those obtained for the same spiked concentrations in deionized water (analyzed both directly and after SPE). The data, presented in Fig. S5 of the Electronic Supplementary Material, reveal that signal suppression is frequently greater (by as much as 50%) in SPE extracts than in wastewater itself, in which mild signal enhancement can occur. This result suggests that the SPE procedure may concentrate interfering chemicals in addition to the target analytes and provides further motivation for conducting analyses via direct injection when possible. This observation also validates the use of stable isotopically labeled standards, as is recommended in methods that are subject to such matrix interferences [43].

Drugs of abuse in wastewater Analyte concentrations in BRWWTP influent are presented in Table 3. Concentrations determined using the primary and confirmatory SPE, RPLC/MS/MS methods, as well as by direct injection LC/MS/MS analysis, are generally in good agreement. The exceptions are norcocaine, norbenzoylecgonine, *m*-hydroxybenzoylecgonine, ecgonine ethyl ester, anhydroecgonine, methamphetamine, and creatinine, for which at least one measurement differs from the others by more than 20%. For norbenzoylecgonine, the variation appears random and is within the experimental error of the three measurements (as defined by their standard deviations). Concentrations determined for the direct injection analysis of ecgonine ethyl ester and *m*-hydroxybenzoylecgonine are close to their respective LODs. This may partially account for the elevated levels reported in Table 3. In contrast, anhydroecgonine and creatinine concentrations are above detection limits for analysis via SPE, LC/MS/MS, but they are among the earliest eluting compounds.

MRM chromatograms are presented in Fig. 3 for the analysis of cocaine and three metabolites (benzoylecgonine, ecgonine methyl ester, and ecgonine) in unamended wastewater via both direct injection and SPE, LC/MS/MS. As demonstrated in chromatograms for ecgonine and ecgonine methyl ester, the increase in background interferences that accompanies early elution underscores the potential pitfalls of analyzing small, highly polar chemicals by RPLC, either by direct injection or with preconcentration via SPE. Also of note is the fact that peak intensities for ecgonine were too low in real wastewater samples to enable analysis via direct injection, even though similar concentrations could be analyzed via direct injection in substitute wastewater.

Matrix effects are another possible source of error, especially for creatinine and norcocaine, as neither has its own isotopically labeled surrogate to account for differences in ionization. Strikingly, even though there can be substantial variation in the concentrations obtained using the three methods, standard deviations for each method are quite low—less than 10% for 20 of 23 analytes. This illustrates the central importance of employing confirmatory analyses (in contrast to a single methodology) to investigate any potential measurement bias and to ensure overall measurement quality.

In total, 21 of 23 analytes (including cocaine and all of its metabolites) were detected in BRWWTP influent using the full SPE, RPLC/MS/MS method(s), and 16 of 23 analytes (including cocaine and nine of its metabolites) were detected by direct injection LC/MS/MS analysis. Concentrations of cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, and norcocaine are all within the range of previously reported values for wastewater influent, as are concentrations of the opioid drugs, phenylamine drugs, and human-use markers [9, 11]. These are the first reported measurements of ecgonine methyl ester, ecgonine ethyl ester, anhydroecgonine methyl ester, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine, ecgonine, and anhydroecgonine in an environmental sample. Interestingly, ecgonine methyl ester has been sought in Belgian wastewater, but never found [10, 15]. In this study, ecgonine methyl ester was detected by all three methods of analysis at concentrations exceeding 400 ng/L. Combined, cocaine, benzoylecgonine, ecgonine methyl ester, and ecgonine represent 91.7±0.2% of the total measured cocaine load in BRWWTP influent. Ecgonine alone represents 28.2±0.5% of that load.

Acknowledgements We wish to thank Aaron Peck and Rebecca Heltsley of NIST for their initial efforts in LC/MS/MS method development. We also thank William Jeffrey (former NIST Director) for bringing this measurement need to our attention.

Conflicts of interest Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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