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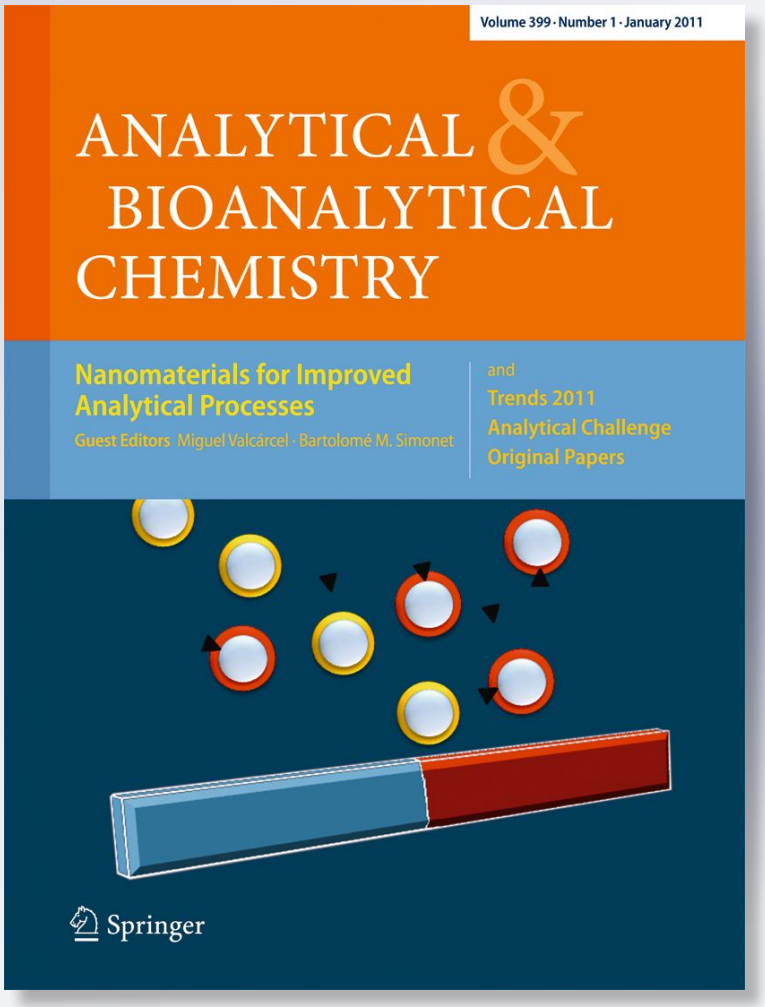

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ORIGINAL PAPER

A hydrolysis procedure for the analysis of total cocaine residues in wastewater

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Abstract We report a sample pretreatment approach for the analysis of total cocaine residues in wastewater that eliminates the need for two key assumptions often made in estimating cocaine utilization from measurement of its benzoylecgonine metabolite: that benzoylecgonine is neither degraded nor generated during transport in a sewer system, and that it is excreted as a constant fraction of cocaine ingested. By adding NaOH and incubating samples at 55 °C, cocaine and its principal metabolites are efficiently hydrolyzed into ecgonine, anhydroecgonine, and norecgonine. Ecgonine, estimated to represent between 37% and 90% (on a molar basis) of cocaine residues, can be directly determined (without preconcentration via solid-phase extraction (SPE)) by reversed-phase (RP) or hydrophilic interaction liquid chromatography–tandem mass spectrometry (LC/MS/MS). If samples are subjected to SPE, anhydroecgonine can also be determined; this metabolite (and its precursors) represents ≈7% of urinary cocaine residues (based on spot collections from living individuals). Although a reference standard for norecgonine is not commercially available, such nortropanes are also a minor fraction (up to 2%) of urinary cocaine residues. The stability of two human markers (cotinine and creatinine) to the hydrolysis procedure was also investigated. Results obtained by applying the hydrolysis approach for the analysis of total cocaine in an untreated municipal wastewater sample (obtained from Baltimore, MD) were generally in excellent agreement with those obtained from split samples analyzed using a more comprehensive solid-phase extraction RPLC/MS/MS method as described in our previous work. In particular, total tropane-based cocaine residues were found to be hydrolyzed to ecgonine with 98–99% efficiency.

Keywords HILIC · Cocaine · Ecgonine · Anhydroecgonine · Hydrolysis · Transformation

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Introduction

Estimates of utilization of drugs of abuse via wastewater analyses (known as sewer epidemiology) are becoming ever more common within municipalities, primarily in Europe [1–13] but also within North America [13–16] and Australia [17, 18]. Sewer-derived estimates of cocaine utilization are typically based on measurements of one key metabolite (usually benzoylecgonine), and assume it is excreted at a constant fraction within a population, despite the fact that data reveal considerable variability [16, 18–23]. Sewer-derived estimates of cocaine utilization moreover assume that benzoylecgonine is neither generated nor degraded during sewer transport and sample processing. Yet

cocaine and its ester-containing metabolites are known to hydrolyze readily in water at ambient temperature and circum-neutral pH [24–26]. Bacterially mediated hydrolysis may accelerate such transformations in municipal wastewater [27]. Indeed, others [8] have observed the ratio of cocaine to benzoylecgonine to decrease in the order of untreated sewage>treated sewage>receiving waters, potentially indicative of environmental transformations. Failure to accurately account for such hydrolysis, or individual variations in metabolic excretion, may introduce bias and increase uncertainty if wastewater-derived estimates of cocaine utilization are based on measurements of a single metabolite [28].

Expanding analytical methods to capture a larger fraction of the total cocaine load entering sewer systems provides one means of reducing uncertainties that might arise from basing estimates of utilization on a single metabolite. We recently developed [29] a comprehensive reversed-phase tandem mass spectrometric (RPLC/MS/MS) method that affords analysis of cocaine and 11 different cocaine metabolites, 9 other drugs of abuse, and 2 human-use markers. Although this approach enables the analysis of some specific cocaine metabolites (e.g., cocaethylene and anhydroecgonine methyl ester) that provide information regarding routes of administration, it suffers from long run times and, for several metabolites, from the need to preconcentrate samples via solid-phase extraction (SPE).

An alternative approach might be to take advantage of the propensity of cocaine and some of its metabolites to hydrolyze by converting cocaine and its ester-containing metabolites to their tropane, nortropane and 2-tropene alkaloid skeletons (Fig. 1). This approach would enable researchers to monitor the total cocaine load in municipal wastewaters through the analysis of only three compounds: ecgonine, anhydroecgonine, and norecgonine. Even if only

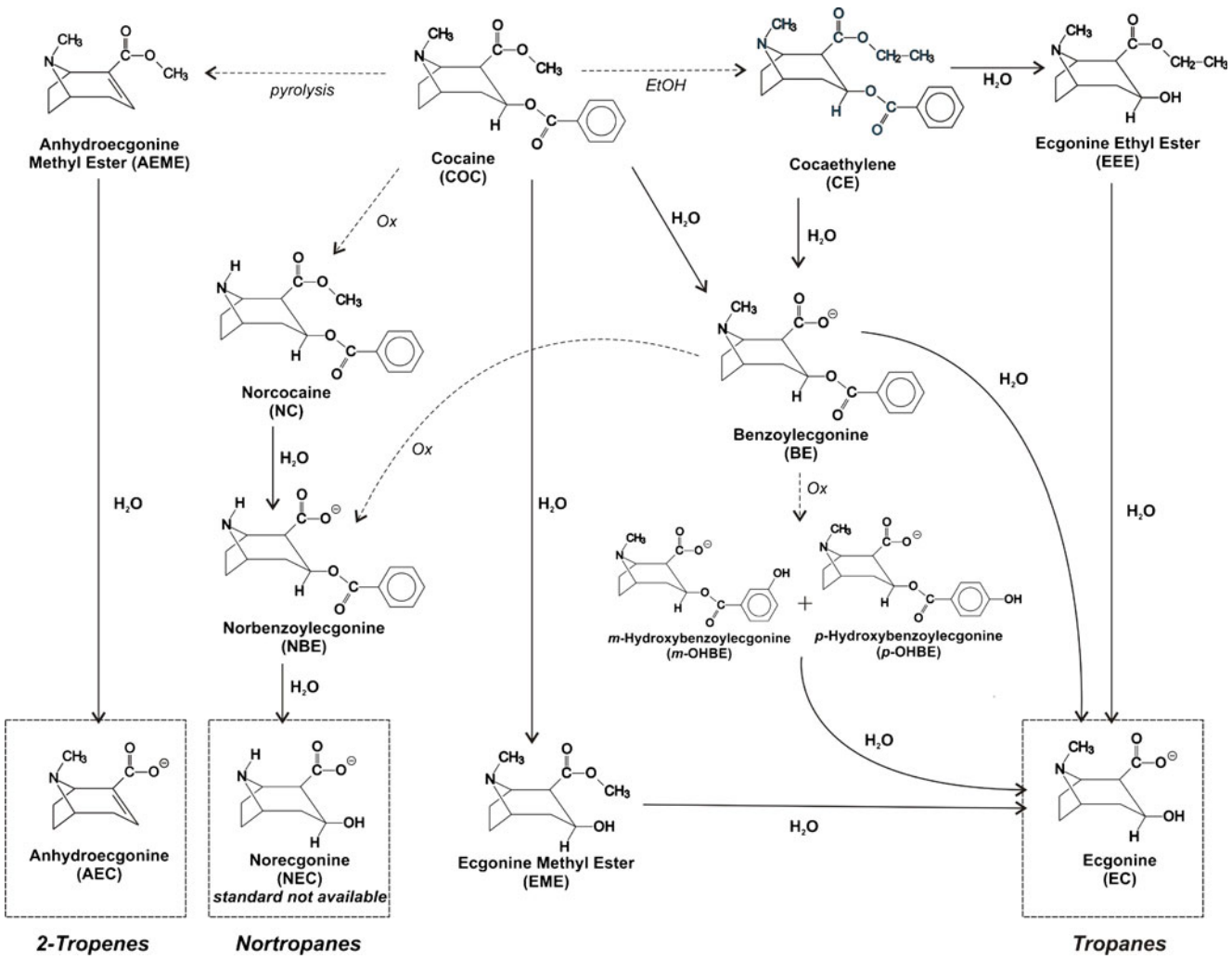

Fig. 1 Schematic for the hydrolysis of cocaine and its major metabolites to their three primary alkaloid skeletons (tropanes, nortropanes and 2-tropenes). *Solid lines* represent hydrolysis reactions that are likely to occur in aqueous solution, while *dashed lines* represent reactions that occur during metabolism (*ox* oxidation; "EtOH" represents transesterification that occurs when cocaine is co-ingested with ethanol) or during administration (e.g., pyrolysis when administered as crack cocaine)

ecgonine were analyzed, a more robust estimate of total cocaine utilization could be derived than is afforded by measurement of benzoylecgonine alone, although some disagreement currently exists between studies as to the fraction of cocaine that is excreted as ecgonine and its precursors. For example, one recent review of dosing studies indicated that ecgonine and its precursors could range between 37% and 75% (on a molar basis) of cocaine metabolites in human urine [16] while another study involving spot collection specimens [23] reported this fraction was as high as 90%. The fraction excreted as tropanes does appear to depend on the route of administration, and may be as low as 37% in the case of smoked ("crack") cocaine [16]. If anhydroecgonine (the hydrolysis product of the cocaine pyrolysis product, anhydroecgonine methyl ester; Fig. 1) were also included as an analyte, route of administration information could still be retained.

Although hydrolysis may proceed relatively slowly at near-neutral pH, Garrett and Seyda [24] and Garrett et al. [25] report that hydrolysis half lives for cocaine, benzoylecgonine, and ecgonine methyl ester in water at pH 12 are less than 30 min at temperatures above 30 °C, implying that hydrolysis could be rapidly effected at elevated pH. The alkaloid skeletons of cocaine and its analogs should be resistant to hydrolysis; moreover, ecgonine and anhydroecgonine have recently been demonstrated to be stable in municipal sewage [28].

Several advantages may result from hydrolyzing cocaine metabolites to ecgonine, anhydroecgonine, and norecgonine. Doing so may increase their concentrations to the point where quantification via direct injection RPLC/MS/MS or even (SPE)-RPLC/MS could be possible. This is especially likely for ecgonine, as it represents the principal hydrolysis end-product of cocaine and six cocaine metabolites, including benzoylecgonine, that all possess a tropane (*N*-methyl-8-azabicyclo[3.2.1]octane) skeletal structure (Fig. 1). In cases where only total cocaine loads are required, circumventing SPE will substantially reduce the required analytical effort. Another advantage to incorporating a hydrolysis step would be realized in analyses where cocaine (and its metabolites) represented the only drug of abuse of interest; in such situations, chromatographic run times could be greatly reduced (from nearly 28 min to less than 4 min using RPLC). Hydrolysis may enable analysis via hydrophilic interaction (HILIC) chromatography, an approach that could potentially improve detection limits for small organic analytes by more strongly retaining them, and by allowing ionization to occur in a less aqueous matrix [30]. HILIC does suffer some disadvantages in effecting separation of a large number of analytes that are structurally similar. For example, in our earlier work [29], we were unable to effect separation of cocaine and 11 of its metabolites using HILIC chromatography. This disadvantage might be eliminated if, through hydrolysis, we could reduce their number to only ecgonine, anhydroecgonine, and (perhaps) norecgonine. Use of HILIC chromatography in such a case may afford a highly sensitive yet rapid approach for the quantitation of total cocaine residues. Finally, and perhaps more importantly, by collapsing a very large fraction of cocaine and its metabolites to a very few quantifiable metabolites, estimates of cocaine utilization can be made without relying on assumptions concerning the fraction of cocaine excreted as any one particular metabolite.

In the present paper, we present a hydrolysis procedure for the analysis of total cocaine residues in municipal wastewater. This sample preparation technique collapses cocaine and its principal metabolites to ecgonine, anhydroecgonine, and norecgonine, thereby minimizing analytical effort without increasing measurement uncertainty or substantially compromising accuracy. We also report on HILIC conditions suitable for the analysis of ecgonine and anhydroecgonine in hydrolyzed wastewater samples. Cotinine and creatinine are commonly included in sewer epidemiology studies as human-use markers whose concentrations might enable correction factors for dilution of human urine in sewage; the influence of the hydrolysis procedure on these two compounds was, therefore, also investigated. We apply this hydrolysis procedure to the analysis of untreated sewage, and compare the results with those we have recently reported (from splits of samples analyzed in the present study) that were based on a more comprehensive (SPE)-RPLC/MS/MS method [29]. Although this approach will not differentiate between ingested cocaine and cocaine disposed directly to sewer systems, any increase in error in computed per capita ingestion of cocaine is counterbalanced by the decrease in uncertainty associated with such estimates that is afforded by capturing a broader array of metabolites [28].

Materials and methods

Chemicals Details on the procurement and handling of analyte and isotopically labeled surrogate standards, as well as the preparation of spiking and calibration solutions, are presented elsewhere [29].

Development of the hydrolysis procedure The stability of cocaine and the 11 cocaine metabolites (molecular structures are presented in Fig. 1) and two human-use markers creatinine and cotinine were investigated under alkaline conditions in deionized water at 22 and 55 °C, and in NaOH-amended municipal wastewater at 55 °C. Grab samples of untreated wastewater were obtained from the Back River Wastewater Treatment Plant (BRWWTP) in Baltimore, MD on February 13, 2009; these were used in

developing the hydrolysis procedure. The BRWWTP serves approximately 944,000 people in the greater Baltimore area. Samples were stored on dry ice and were then filtered through 1.2 μm Millipore GF/C filters (Bedford, MA) within 6 h after collection. Aliquots (4 mL in capped test tube reactors) of water or wastewater were brought to the appropriate temperature in heated water baths, and were spiked with a solution containing all analytes to obtain a resulting concentration of between 100 and 200 μg/L per analyte (resulting in a minimum of 38× background levels for the wastewater reactors).

After taking an initial 200-μL sample, the deionized water reactors were adjusted to 0.020 M NaOH and the wastewater reactors were adjusted to 0.025 M NaOH. These concentrations had previously been determined to suffice to raise the pH of the respective solutions to 12.3 at room temperature. The reactors were then sampled by removing a 200 μL aliquot at 15, 30, 60, 90, 120, and 180 min (the last for the wastewater reactors only), while inverting reactors intermittently to provide mixing. All samples were immediately amended with sufficient HCl to lower the pH to approximately 2.0 (at room temperature). The final HCl concentration was 0.030 M in deionized water samples and 0.036 M in wastewater samples. The samples were then amended with acetonitrile containing our limited suite of isotopically labeled surrogates ("Group B"; see Table S1 in the Electronic Supplementary Material) to provide a final concentration of 70 μg/L per surrogate, and were stored at −20 °C until analysis. Investigations in deionized water were performed in duplicate, and investigations in wastewater were performed in triplicate. All samples were analyzed without further processing by a direct injection RPLC/MS/MS technique; details regarding this method and its validation, including quality assurance and reproducibility, are provided elsewhere [29]. Instrument detection limits (IDLs) and limits of detection (LODs) obtained from this and related methods are summarized in Table S2 in the Electronic Supplementary Material.

Application to total cocaine residues in wastewater A 24-h, flow-weighted composite sample (4 L) of wastewater influent (post-screening) was collected from the BRWWTP in Baltimore, MD on April 30, 2009. The plant flow on the day of sample collection was 405×10^6 L/d. The sample was transported to our laboratory on dry ice, whereupon it was split into four subsamples (Fig. 2). Two subsamples

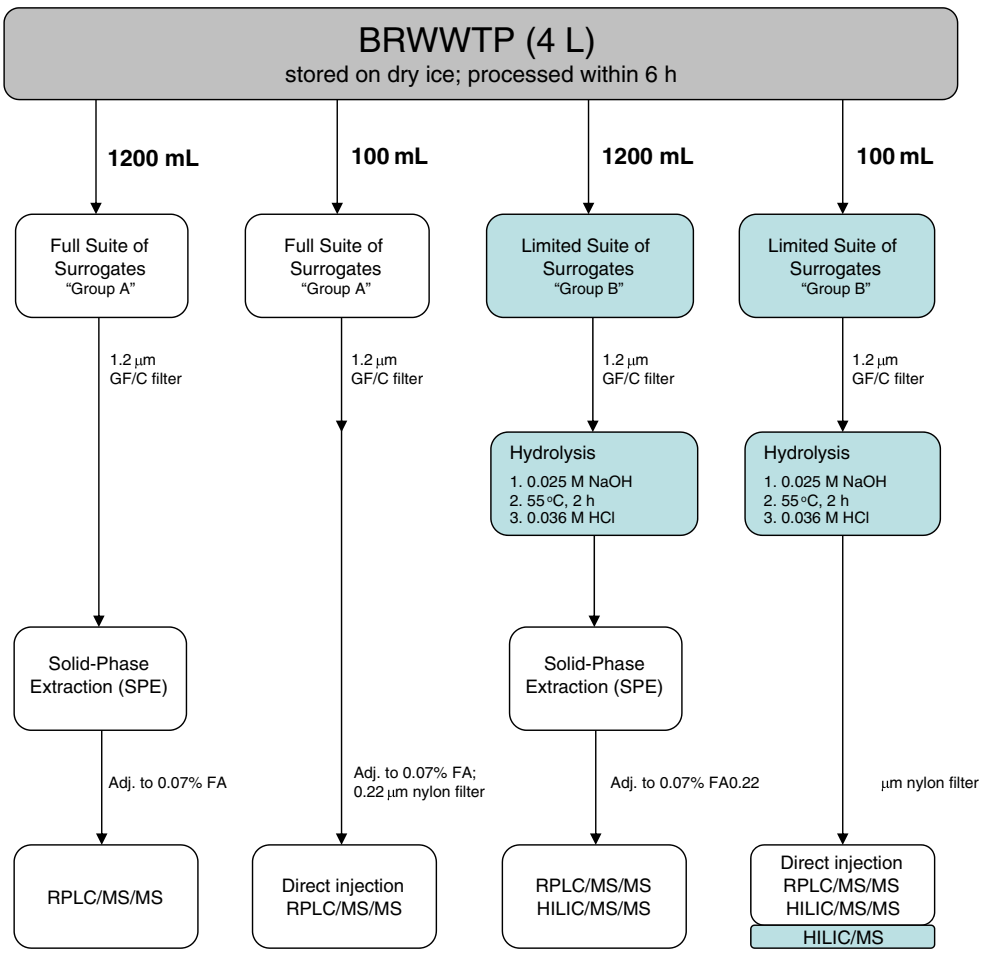

Fig. 2 Overview of the Back River Wastewater Treatment Plant (BRWWTP) influent sample processing and analytical methodology. Unique steps relevant to the hydrolysis protocol that are the focus of this manuscript are highlighted in blue. "Group A" and "Group B" surrogates are defined in Table S1 in the Electronic Supplementary Material

were analyzed via RPLC/MS/MS, with and without preconcentration via SPE, and without being subjected to hydrolysis; results of these analyses have been reported in our previous work [29]. The remaining two subsamples were amended with acetonitrile containing our limited suite of isotopically labeled surrogates ("Group B"; Table S1 Electronic Supplementary Material) then subjected to hydrolysis, and were then analyzed via RPLC/MS/MS, as well as via HILIC/MS/MS and HILIC/MS. One of these subsamples was concentrated via SPE before analysis, while the other was analyzed by direct injection. Further details on the analytical procedure are provided in the Electronic Supplementary Material.

LC separations An injection volume of 5 μL was used in all LC analyses. RPLC separations for unhydrolyzed, partially hydrolyzed, and fully hydrolyzed samples were achieved on a Restek Viva pentafluorophenyl-propyl column (2.1×150 mm, 5 μm) 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.

HILIC separations were performed to analyze ecgonine and anhydroecgonine only, and were achieved on a Restek Ultra IBD phase (2.1×150 mm, 5 μm) column and guard column (2.1×10 mm, 5 μm) at 55 °C, using 10 mmol/L ammonium formate (pH 2.9) and acetonitrile (containing 0.1% (volume fraction) formic acid) as the mobile phase (200 μL/min). The acetonitrile content was decreased linearly from 95% to 5% over 5 min, and was then held at 5% for 5 min. The column was re-equilibrated at 95% acetonitrile for 30 min between runs. This fast gradient condition was employed in an effort to minimize run times and improve peak shape, thus ionization occurred for both analytes under highly aqueous conditions. It is important to note that ecgonine and anhydroecgonine can also be eluted isocratically at 95% acetonitrile, but substantial peak spreading occurs under these conditions, making it unlikely that detection limits will be improved.

MS/MS and MS quantitation LC/MS/MS analyses were conducted using an Agilent 1200 series Binary LC System (Palo Alto, CA) coupled to an API 5000™ triple quadrupole mass spectrometer and Turbo V™ Ionspray source (Applied Biosystems Inc., Foster City, CA). Both the RPLC and HILIC analyses were performed using previously optimized MS/MS instrument parameters [29]. Additional MS/MS quantitation details and a description of the surrogate standards used in the analyses are provided in the Electronic Supplementary Material.

Ecgonine and anhydroecgonine analyses were also attempted using direct injection HILIC/MS, using an Agilent 1100 Series single quadrupole LC-MSD with vacuum degasser, autosampler, and electrospray source operating in positive ionization mode. Capillary voltage (4,000 V), nebulizer pressure (446 kPa), drying gas flow (12 L/min) and temperature (350 °C) were optimized under LC flow conditions (200 μL/min). Optimized fragmentation voltages of 125 V and 100 V were used for ecgonine and anhydroecgonine, respectively.

Results and discussion

Hydrolysis procedure development Time courses for cocaine, cocaethylene, and their tropane metabolites are presented in Fig. 3 for deionized water and amended wastewater under alkaline conditions (55 °C; ≥ 0.020 M NaOH). Error bars associated with each data point represent the standard deviation of results from replicate reactors (n= 2 for deionized water and n=3 for wastewater). With the exception of *p*-hydroxybenzoylecgonine, all ester-containing tropanes exhibited rapid degradation in both water and wastewater at 55 °C. This degradation was accompanied by a stoichiometric increase in ecgonine concentrations, such that excellent (>95%) mass balances on the tropane backbones were obtained. Together, these observations provide strong evidence that hydrolysis occurred as outlined in Fig. 1. They also demonstrate that 2 h of reaction at 55 °C and 0.025 M NaOH suffices to quantitatively convert cocaine, cocaethylene, and most of their alkyl and benzoyl ester metabolites to ecgonine in a matrix as complex as municipal sewage, and that ecgonine itself is stable under these reaction conditions.

m- and *p*-Hydroxybenzoylecgonine hydrolyzed more slowly than their unsubstituted analogs. The *para*-substituted hydroxybenzoylecgonine hydrolyzed more slowly than the *meta*-substituted isomer, with greater than 80% of the former remaining after 3 h of reaction at 55 °C. Hydroxy substituents in the *para* position inhibit the hydrolysis of benzoyl esters through a combination of resonance and inductive effects, while hydroxy substituents in the *meta* position only exert inductive effects [31]. The more pronounced influence of the *p*-OH substituent on hydrolysis rates is consistent with its more negative Hammett constant (σ_p=−0.335) compared to that of the *meta* hydroxy substituent (σ_m=−0.018) [32]. Fortunately, because *p*-hydroxybenzoylecgonine only represents approximately <1.0% and 1.4% of the total measured cocaine load in human urine [16] and in wastewater [29], respectively, its incomplete conversion should only exert a

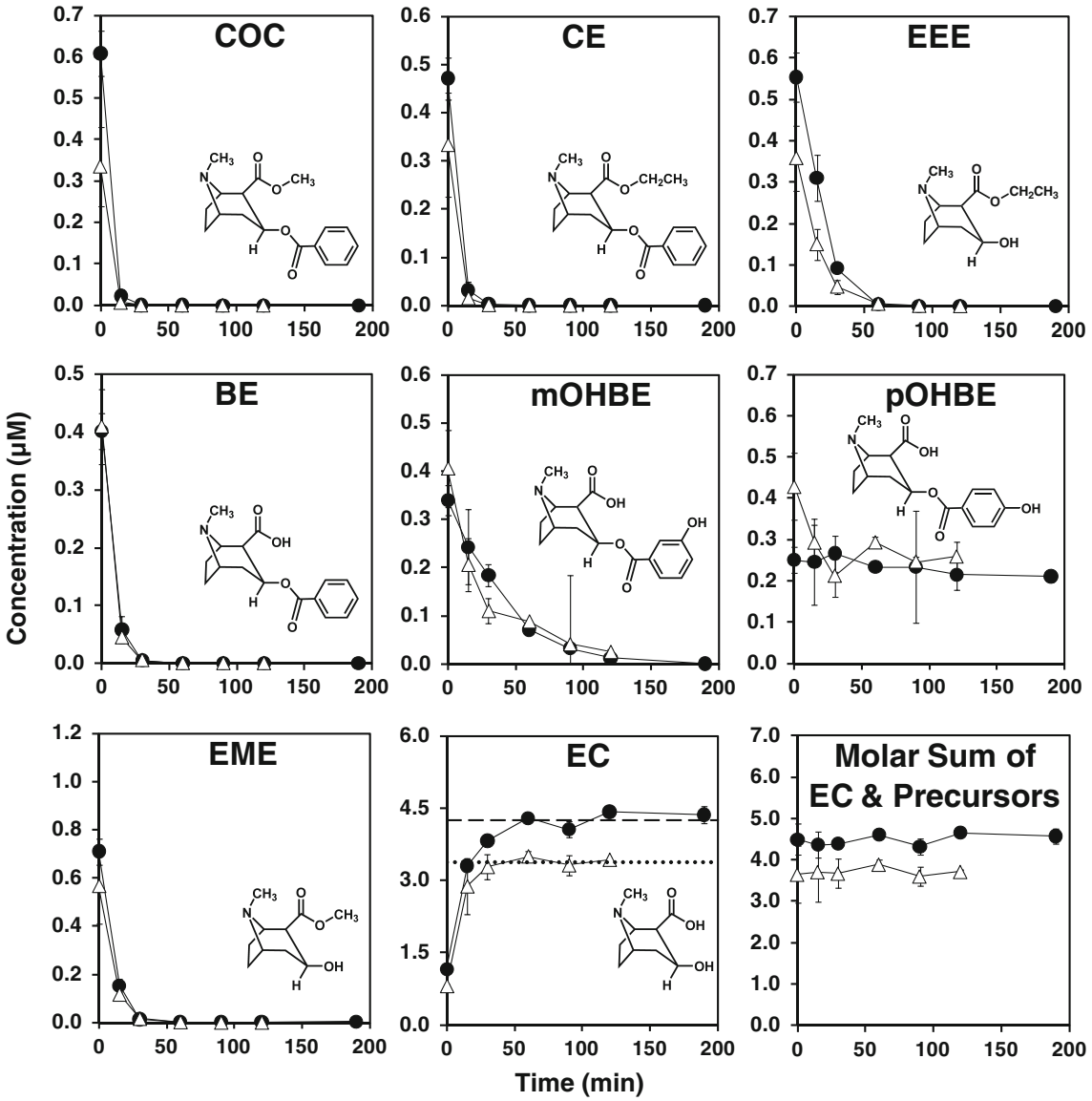

Fig. 3 Hydrolysis of cocaine, cocaethylene, and their metabolites to ecgonine (*EC*) at 55 °C in deionized water containing 20 mmol/L NaOH (*open upright triangle*), and in amended wastewater containing 25 mM NaOH (*filled circle*). The *dashed* (– – – –) and *dotted* (·····) lines in the EC plot represent the predicted, post-hydrolysis concentrations of ecgonine in wastewater and deionized water, respectively. *Error bars* represent one standard deviation on samples from replicate reactors (n=2 for deionized water and n=3 for wastewater). Refer to Fig. 1 for abbreviations

minor impact on post-hydrolysis ecgonine measurements in environmental samples.

The stability of the cocaine pyrolysis metabolites (2-tropenes) at 55 °C and ≥0.020 M NaOH is presented in Fig. 4. Anhydroecgonine methyl ester was quantitatively converted to anhydroecgonine in both water and wastewater after approximately 1 h. The total mass of the 2-tropene backbone was conserved during the hydrolysis procedure, though an increase in variance was unexpectedly observed for the MS/MS analysis of anhydroecgonine in the deionized water samples. As with cocaine and cocaethylene, these results indicate that the hydrolysis procedure fully converts anhydroecgonine methyl ester to its 2-tropene backbone (anhydroecgonine) according to the pathway presented in Fig. 1. The results also suggest that anhydroecgonine is stable under alkaline conditions in municipal wastewater.

Norcocaine and norbenzoylecgonine, the nortropane metabolites, were completely degraded within 15 min under the conditions tested (Fig. 5). Norecgonine, monitored in the wastewater reactors only, was formed in parallel with the disappearance of norcocaine and norbenzoylecgonine. Because no commercially available standard exists for norecgonine, its formation could only be semi-quantitatively assessed; concentrations reported in Fig. 5 assume that the

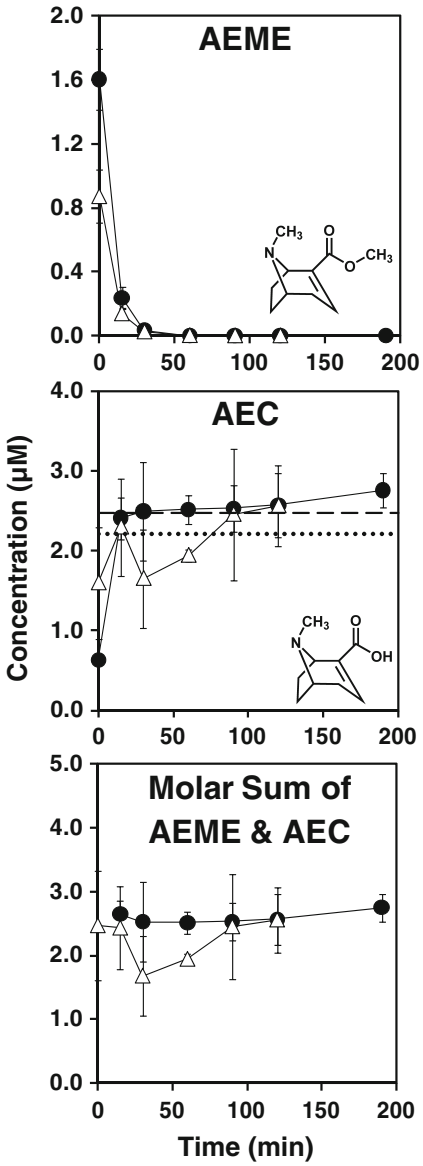

Fig. 4 Hydrolysis of the cocaine pyrolysis products anhydroecgonine methyl ester (*AEME*) to anhydroecgonine (*AEC*) at 55 °C in deionized water containing 20 mmol/L NaOH (*open upright triangle*), and in amended wastewater containing 25 mmol/L NaOH (*filled circle*). The *dashed* (– – – –) and *dotted* (·····) lines in the AEC plot represent the predicted, post-hydrolysis concentrations of anhydroecgonine in wastewater and deionized water, respectively. *Error bars* represent one standard deviation on samples from replicate reactors (n=2 for deionized water and n=3 for wastewater)

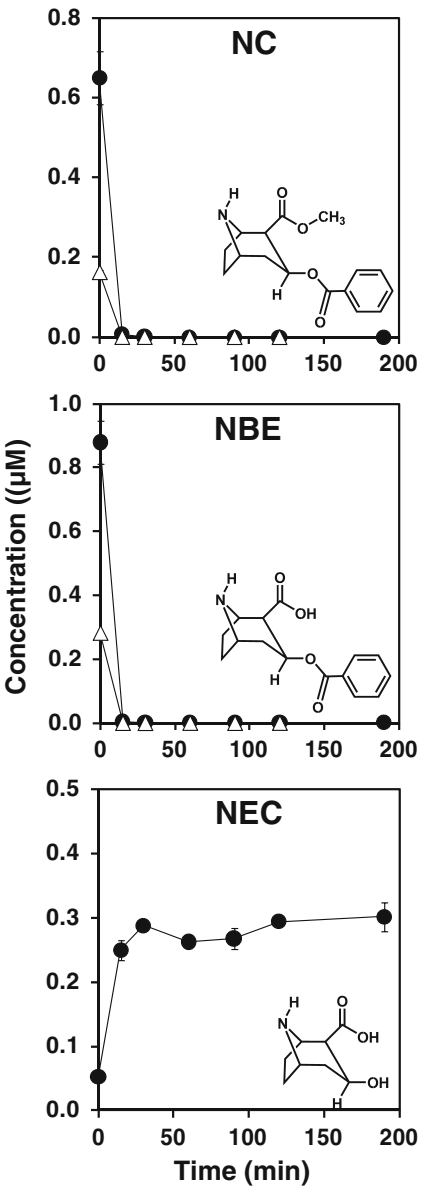

Fig. 5 Hydrolysis of norcocaine (*NC*) and norbenzoylecgonine (*NBE*) to norecgonine (NEC) at 55 °C in deionized water containing 20 mmol/L NaOH (*open upright triangle*), and in amended wastewater containing 25 mmol/L NaOH (*filled circle*). Note that reported concentrations for NEC are only semi-quantitative estimates, as an authentic standard for this compound is not available. *Error bars* represent one standard deviation on samples from replicate reactors (n =2 for deionized water and n=3 for wastewater)

molar response of norecgonine is identical to that of ecgonine. Norcocaine and norbenzoylecgonine appear to be degraded via the pathway outlined in Fig. 1, and norecgonine appears stable under alkaline conditions in municipal wastewater. The extent of conversion of norcocaine and norbenzoylecgonine to their nortropane backbones cannot, however, be quantitatively assessed without an authentic standard for norecgonine. Fortunately, *N*-demethylation is a minor pathway in the human metabolism of cocaine (*N*-demethylated metabolites comprise on average 2% of measured urinary cocaine loads from spot collections from living individuals [23] and at most 1.2% for administered dose studies [16]). It is therefore assumed that norecgonine can be excluded from analysis with only a minor reduction in the total amount of cocaine residues determined after hydrolysis.

The stability of two human-use markers (cotinine and creatinine) to the hydrolysis procedure was also investigated.

Cotinine is a major urinary metabolite of nicotine [33], and creatinine is used to identify the presence of human urine in wastewater analysis [14]. Time courses for these analytes in water and wastewater at 55 °C and ≥0.020 M NaOH are presented in Fig. S1 in the Electronic Supplementary Material. Some loss in concentration in either water or wastewater is exhibited, though in each case the observed loss is within or close to the experimental error (as quantified by the standard deviation of replicate samples).

Application to cocaine residues in municipal wastewater The hydrolysis procedure was applied to the analysis of a 24 h, flow-weighted composite sample of BRWWTP wastewater influent. Concentrations of the hydrolysis products of cocaine and cocaethylene are presented in Table 1. The hydrolysis procedure quantitatively converts cocaine and its esterified tropane metabolites to ecgonine with near 100% efficiency. A comparison of the total ion chromatogram and extraction ion chromatograms for the analysis of ecgonine in wastewater post-hydrolysis for both the HILIC and RPLC analyses is shown in Fig. 6. Also reported in Table 1 are results of SPE-RPLC/MS/MS analyses of cocaine, cocaethylene, and their metabolites in split samples that were not subjected to hydrolysis, as taken from our prior work [29]. Neither cocaine, nor any of its ester-containing metabolites, could be detected in hydrolyzed samples by RPLC/MS/MS, with or without SPE, although an unidentified interference prevented accurate quantification of *p*-hydroxybenzoylecgonine. This interference did not affect *m*-hydroxybenzoylecgonine, and was not present during preliminary analyses of *p*-hydroxybenzoylecgonine in hydrolyzed wastewater.

Two of the three anticipated hydrolysis products (ecgonine and anhydroecgonine) could be detected in the

Table 1 Concentrations of cocaine residues in unamended Back River Wastewater Treatment Plant (BRWWTP) influent

	Unhydrolyzed[a]	Hydrolyzed			
Target analytes	RPLC SPE	RPLC SPE[b]	RPLC Direct[c]	HILIC SPE[d]	HILIC Direct[e]
Tropanes					
Cocaine	2.66±0.005	ND[f]	ND	–	–
Benzoylecgonine	9.31±0.17	ND	ND	–	–
Ecgonine methyl ester	2.07±0.02	ND	ND	–	–
p -Hydroxybenzoylecgonine	0.18±0.004	Unk[g]	Unk[g]	–	–
m-Hydroxybenzoylecgonine	0.06±0.01	ND	ND	–	–
Cocaethylene	0.06±0.004	ND	ND	–	–
Ecgonine ethyl ester	0.16±0.01	ND	ND	–	–
Ecgonine	6.22±0.21	21.0±0.63	22.3±1.3	20.4±1.0	20.5±0.89
Subtotal:	20.7±0.22				
2-Tropenes					
Anhydroecgonine methyl ester	0.08±0.003	ND	ND	–	–
Anhydroecgonine	0.55±0.02	0.35±0.11	<LOD[h]	<LOD	<LOD
Subtotal:	0.58±0.02				
Nortropanes					
Norcocaine	0.04±0.001	ND	ND	–	–
Norbenzoylecgonine	0.71±0.09	ND	ND	–	–
Norecgonine	ND	ND	ND	–	–
Subtotal	0.75±0.09				
Total	22.0±0.24	21.4±0.64	22.3±1.3	20.4±1.0	20.5±0.89

Average concentrations (±1 standard deviation) are presented in nmol/L to facilitate comparison

[a] Results from Ref. [29]; analyses conducted via SPE-RPLC/MS/MS using a pentafluorophenyl-propyl (PFPP) column (n=5)

[b] SPE-RPLC/MS/MS analysis of hydrolyzed samples using the PFPP column (n=5)

[c] RPLC/MS/MS analysis (without SPE) of hydrolyzed samples using the PFPP column (n=8)

[d] SPE-HILIC/MS/MS analysis of hydrolyzed samples using the IBD column (n=5)

[e] HILIC/MS/MS analysis (without SPE) of hydrolyzed samples using the IBD column

[f] Not detected

[g] Experimental artifacts prevented accurate quantitation

[h] <LOD: Less than the limit of detection (LODs are listed in Table S2 in the Electronic Supplementary Material)

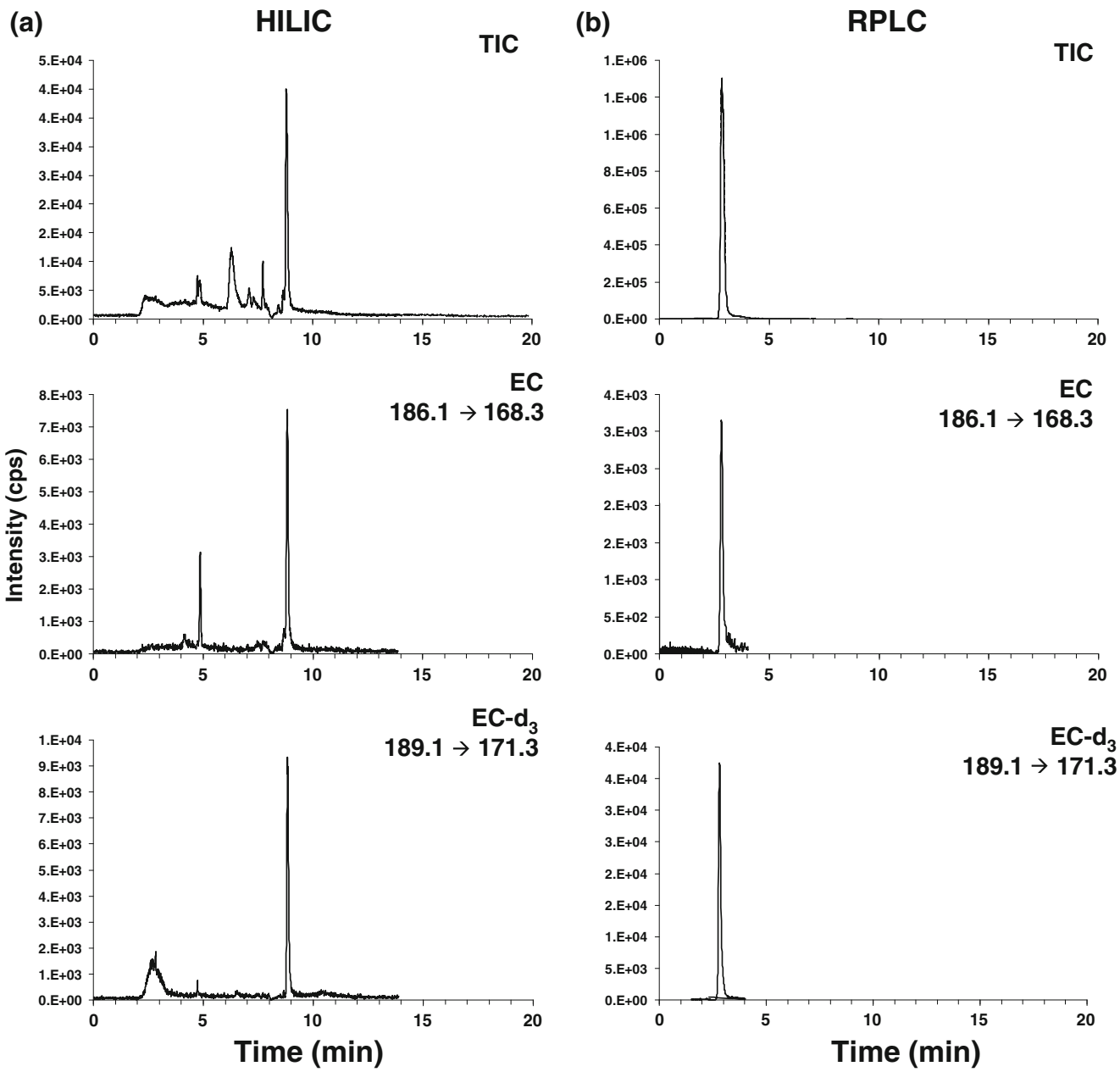

Fig. 6 Total ion chromatograms (TIC) and extracted ion chromatograms for ecgonine (EC) and ecgonine-d_3 (EC-d_3) in wastewater (post-hydrolysis) for both **a** HILIC and **b** RPLC separation methods

hydrolyzed wastewater samples. The presence of the remaining hydrolysis product (norecgonine) could not be verified, owing to the lack of an authentic standard. The hydrolysis procedure increased ecgonine concentrations to approximately 20 nmol/L, well above its IDL value and estimated LOD (Table S2 Electronic Supplementary Material), allowing it to be quantified by direct injection as well as by SPE-RPLC/MS/MS. Ecgonine concentrations in hydrolyzed samples determined by direct injection or after concentration via SPE agree to within their respective standard deviations (less than 10%). As quantified by SPE-RPLC/MS/MS, the concentration of anhydroecgonine measured in hydrolyzed samples (0.35 nM) is only slightly above the estimated LOD (0.1 nmol/L; Table S2 Electronic Supplementary Material). Notably, this post-hydrolysis concentration of anhydroecgonine was too low to afford its determination by direct injection. The amount of anhydroecgonine measured in hydrolyzed samples could account for (at most) 80% of the amount of anhydroecgonine and its methyl ester measured in the non-hydrolyzed wastewater sample, even though anhydroecgonine was observed to be stable under alkaline conditions in the spiked wastewater experiments. Finally, the depletion of norcocaine and norbenzoylecgonine in the hydrolyzed

samples is consistent with hydrolysis of these metabolites, although lacking a reference standard for norecgonine, this cannot be confirmed. Had such hydrolysis occurred, however, the anticipated concentration of norecgonine (≈0.7 nM) would likely be below the LOD for direct injection. This analyte may require preconcentration for accurate quantification. It is important to emphasize that 2-tropene and nortropane metabolites (hydrolyzed to anhydroecgonine and norecgonine, respectively) are present at such relatively low levels (compared to ecgonine) that omission of these analytes will have little effect on estimations of total cocaine utilization.

Ecgonine and anhydroecgonine were also quantified in hydrolyzed samples in HILIC mode using tandem mass spectrometry (MS/MS). Selected reaction monitoring chromatograms for the dominant MRM transition in MS/MS analysis for ecgonine are provided in Fig. 6; comparable data for both ecgonine and anhydroecgonine are presented in Fig. S2 of the Electronic Supplementary Material. Concentrations of ecgonine (anhydroecgonine was below the limit of quantitation) determined by HILIC/MS/MS are in good agreement with values determined by RPLC/MS/MS; the two results have comparable RSDs (Table 1). Although both analytes elute well past the column void volume (k'=4.43 for both), HILIC/MS/MS analysis did not significantly improve the relative signals over analysis by RPLC/MS/MS, although the HILIC method was still able to quantify hydrolyzed ecgonine by direct injection.

Attempts were also made to determine ecgonine and anhydroecgonine by direct injection single quadrupole LC/MS. Estimated detection limits for direct injection HILIC/MS analysis (10,000 ng/L for ecgonine and 25,000 ng/L for anhydroecgonine; Table S2 Electronic Supplementary Material), obtained from calibration standards in deionized water using a signal-to-noise ratio of 3, were an order of magnitude higher than concentrations determined during analysis by direct injection HILIC/MS/MS (approximately 800 ng/L for ecgonine and 2,500 ng/L for anhydroecgonine; Ref. [29] and Table S2 Electronic Supplementary Material). As a result, neither ecgonine nor anhydroecgonine could be detected in hydrolyzed wastewater samples by direct injection HILIC/MS analysis. Although not attempted in the present study, it should be possible to quantify ecgonine by HILIC/MS after sample preconcentration via SPE, as post-hydrolysis concentrations were only a factor of two below the instrument (i.e., direct injection) detection limit.

In summary, the hydrolysis procedure reported herein, followed by either RPLC or HILIC/MS/MS, may be the approach of choice if total cocaine loads (rather than cocaine ingestion) is of interest. Total cocaine loads may provide a metric more closely related to trafficking in illicit drugs than is afforded by estimates of cocaine ingestion. This analysis can be performed via direct injection to determine cocaine residues with reduced analytical uncertainty. Direct injection, even with a hydrolysis step included, reduces sample preparation time and cost relative to techniques that employ SPE; it is also less susceptible to matrix effects [29]. Although inclusion of anhydroecgonine would require preconcentration via SPE, the results would furnish information pertaining to route of administration.

We note that the most appropriate method for the analysis of cocaine and other drugs of abuse in environmental samples will depend on the type of information being sought, as well as the availability of instrumentation. A full RPLC/MS/MS method [29] can provide detailed data for cocaine and 11 metabolites, including several that can be used to assess the route of cocaine administration. Such results may be of value to researchers wishing to evaluate specific patterns of cocaine abuse, or to delineate cocaine consumption via human ingestion (and thus excretion) from total cocaine loadings (the latter including direct discharges of non-ingested cocaine). The hydrolysis procedure described herein captures as much as 90% of cocaine residues through the analysis of a single analyte (ecgonine). Although inclusion of anhydroecgonine would require preconcentration via SPE, the results would furnish information pertaining to route of administration. The analysis of ecgonine and anhydroecgonine can be completed in under 4 and 10 min of RPLC/MS/MS and HILIC/MS/MS chromatographic run time, respectively, compared to 28 min for the RPLC analysis of cocaine, cocaethylene, and 10 of their metabolites as reported in our related work [29]. If the only instrumentation available to a sewer epidemiology study consists of a single quadrupole LC/MS, it should be possible to determine total cocaine residues in wastewater by "collapsing" a very large fraction of cocaine (and its metabolites) to a single analyte, ecgonine, followed by ecgonine concentration via solid-phase extraction. When using the hydrolysis procedure to monitor total cocaine residues, confirmatory measurements can be obtained by complementing RPLC/MS/MS analyses with HILIC/MS/MS or possibly by HILIC/MS, provided that SPE is performed prior to analysis.

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