



## An automated SPE/LC/MS/MS method for the analysis of cocaine and metabolites in whole blood<sup>☆</sup>

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### ABSTRACT

As laboratories are called upon to develop novel, fast, and sensitive methods, here we present a completely automated method for the analysis of cocaine and its metabolites (benzoylecgonine, ecgonine methyl ester, ecgonine and cocaethylene) from whole blood. This method utilizes an online solid-phase extraction (SPE) with high performance liquid chromatographic separation and tandem mass spectrometric detection. Pretreatment of samples involve only protein precipitation and ultracentrifugation. An efficient online solid-phase extraction (SPE) procedure was developed using Hysphere MM anion sorbent. A gradient chromatography method with a Gemini C6-Phenyl (50 mm × 3.00 mm i.d., 5 µm) column was used for the complete separation of all components. Analysis was by positive ion mode electrospray ionization tandem mass spectrometry, using multiple reaction monitoring (MRM) to enhance the selectivity and sensitivity of the method. For the analysis, two MRM transitions are monitored for each analyte and one transition is monitored for each internal standard. With a 30-µL sample injection, linearity was analyte dependent but generally fell between 8 and 500 ng/mL. The limits of detection (LODs) for the method ranged from 3 to 16 ng/mL and the limits of quantitation (LOQs) ranged from 8 to 47 ng/mL. The bias and precision were determined using a simple analysis of variance (ANOVA: single factor). The results demonstrate bias as <7%, and %precision as <9% for all components at each QC level.

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### 1. Introduction

Worldwide, cocaine is one of the most widely abused recreational drugs and thus, is commonly encountered in many forensic and clinical toxicology cases. A good knowledge of the metabolism of cocaine is critical to understanding its pharmacology and in designing appropriate analytical protocols [1–3]. In this study, we have developed and validated an analytical method to quantitatively measure cocaine and four metabolites (benzoylecgonine, ecgonine methyl ester, ecgonine and cocaethylene) in whole blood. Benzoylecgonine (BZE) is one of the major metabolites formed by either spontaneous hydrolysis or by hepatic carboxyesterase enzymes. The other major metabolite is ecgonine methyl ester (EME), which is formed by hepatic cholinesterase [4–6]. Both of these metabolites have long half-lives in biological matrices and are therefore important to test for in biological matrices when cocaine

exposure is suspected. Both BZE and EME are further hydrolyzed to ecgonine (E). When cocaine is co-administered with alcohol, cocaethylene (CE) is formed in the body. Cocaine is rapidly metabolized *in vivo* and *in vitro*, therefore making it critical to test for its metabolites in biological fluids.

The large polarity difference between cocaine and its highly polar metabolites presents challenges in the simultaneous extraction and chromatographic separation of cocaine and metabolites from complex biological matrices. In the past, published literature has shown that analyses were performed using fast screening by immunoassay followed with confirmation by liquid chromatography with ultraviolet detection (LC/UV) [6–10]. The LC/UV methodology does not provide the necessary specificity, and as a result was replaced with gas chromatography/mass spectrometry (GC/MS) combined with derivatization [11–19]. GC/MS has long been the analytical technique of choice for analysis of complex matrices due to its selectivity and sensitivity and is often the reference method for analysis of drugs in biological matrices. However, over the past decade, the use of liquid chromatography/mass spectrometry (LC/MS) has significantly increased for routine analysis because of sensitivity and affordability [20–26].

Throughout the fields of clinical and forensic toxicology, laboratories are facing increasing workloads and demands to provide

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higher quality results at a faster pace. One obvious solution to this demand is to introduce more automation into toxicological analyses. Often when one contemplates automation, the use of robotics may be considered.

The introduction of robotics into toxicological analyses is not new, but their use has generally been limited to autosamplers, without widespread use in sample preparation and extraction. Because of the repetitive actions in performing SPE, it lends itself to fairly easy automation. There have been several systems made commercially where the sample preparation is done offline. But the best concept for full automation occurs when SPE can be directly linked to the GC/MS or an LC/MS. Here we present a novel, fast, sensitive, and highly specific automated method for the analysis of cocaine and its major metabolites in whole blood.

## 2. Experimental

### 2.1. Materials

Certified standards of E, EME, BZE, C and CE were each purchased as 1000 µg/mL solutions from Cerilliant Corporation (Round Rock, TX). Likewise, internal standards, *d*<sub>3</sub>-ecgonine (*d*<sub>3</sub>-E), *d*<sub>3</sub>-ecgonine methyl ester (*d*<sub>3</sub>-EME), *d*<sub>3</sub>-cocaine (*d*<sub>3</sub>-C), and *d*<sub>3</sub>-coacetylene (*d*<sub>3</sub>-CE) were purchased at concentrations of 100 µg/mL from Cerilliant Corporation (Round Rock, TX). A *d*<sub>5</sub>-benzoyllecgonine (*d*<sub>5</sub>-BZE) internal standard was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA) and was prepared at a concentration of 120 µg/mL. Ten different SPE sorbents: CN (cyanopropyl phase), C2 (silica based ethyl phase), C8 (silica based octyl phase), C8-EC (end-capped silica based octyl phase), C18-EC (end-capped silica based octadecyl phase), C18 High Density (end-capped silica based with a high number of octadecyl chains), General Phase (GP) resin (polydivinyl-benzene phase), Strong Hydrophobic (SH) (modified polystyrene divinylbenzene phase), Cation Exchange (Hypsphere MM Cation) and Anion Exchange (Hypsphere MM Anion) were purchased from Spark Holland, Inc. (Emmen, The Netherlands).

Blank whole blood purchased from Clinical Controls International (Los Osos, CA) was certified to be negative for common drugs-of-abuse and prescription medications using existing in-house immunoassay and chromatographic procedures. Zinc sulphate heptahydrate was purchased from EMD Chemical Inc. (Gibbstown, NJ) and was prepared as a 0.2 M solution. Using the 0.2 M zinc sulphate heptahydrate solution, a protein precipitation solution was prepared with methanol at a ratio of 20:80. Sodium phosphate monobasic monohydrate and sodium phosphate dibasic heptahydrate were purchased from Fisher Scientific and were used to prepare a 0.1 M buffer solution (pH 6.0). Ultrafree-CL centrifugal filter devices were purchased from Millipore Corporation (Bedford, MA). Acetonitrile, methanol, and isopropanol were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid was purchased from Aldrich (Milwaukee, WI). De-ionized water (>18 MΩ grade) was obtained from an in-house Millipore purification system. High performance liquid chromatography columns used in the study were purchased from Phenomenex Inc. (Torrance, CA) and Waters Corporation (Franklin, MA). From the group of columns investigated, the Gemini C6-Phenyl and the Xterra C18 (50 mm × 3.00 mm i.d., 5 µm) provided optimal separation of all the components. The Gemini C6-Phenyl was selected for method validation and used in this procedure.

### 2.2. Procedure

Twelve-point calibration curves (4, 10, 20, 30, 50, 100, 200, 240, 300, 340, 400 and 500 ng/mL) were prepared in whole blood from

a 1 µg/mL combined (E, EME, BZE, C, CE) intermediate whole blood stock standard. An independent solution of 1 µg/mL in whole blood was prepared daily from a QC stock solution of 1000 µg/mL for E, EME, BZE, C, and CE. The 1 µg/mL-QC solution and QC standards at 50, 200 and 400 ng/mL were prepared daily in triplicate. 10 µL of a 10 µg/mL combined internal standard solution (*d*<sub>3</sub>-E, *d*<sub>3</sub>-EME, *d*<sub>3</sub>-C, *d*<sub>3</sub>-CE) and 10 µL of a 12 µg/mL *d*<sub>5</sub>-BZE solution were added to all samples. Blank blood samples were also analyzed with no internal standard added. Pretreatment of the samples to precipitate plasma proteins was carried out by adding 1 mL of zinc sulphate heptahydrate/methanol solution to half of a milliliter of each blood sample. The samples were then vortexed well and centrifuged for 15 minutes at 4000 rpm. Half of a milliliter of the supernatant was removed and transferred to a labeled centrifuge tube with a filter. Half of a milliliter of phosphate buffer was added and the tubes were centrifuged at approximately 3000 rpm for 5 min. Upon completion, filtered samples were transferred to autosampler vials. A partial loop injection of 30 µL of the filtered, diluted sample was made.

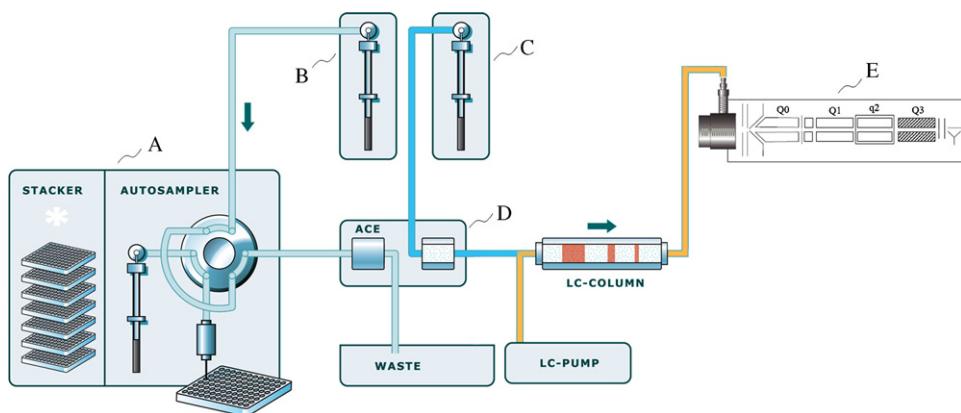
### 2.3. Instrumentation

This automated method is unique in that it interfaces the Spark Holland Symbiosis™ (Emmen, The Netherlands) to the Applied BioSystems 4000Qtrap (Ontario, Canada) so that these two instruments function as a single unified system (Fig. 1). The Symbiosis is comprised of a refrigerated storage compartment (maintained at 12 °C), an autosampler, a solid phase extraction unit (automated cartridge exchanger (ACE)), a solvent delivery unit consisting of two high-pressure dispensing pumps (HPD), and two high performance liquid chromatography (HPLC) pumps. The ACE module contains two connectable 6-way valves and an SPE cartridge exchange module. The HPD provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump is a binary high-pressure gradient pump. The Hysphere MM Anion exchange (10 mm × 2 mm) SPE cartridge was used for the sample extraction, while a Gemini C6-Phenyl column was used for chromatographic separation. The column temperature was at ambient room temperature. Detection was performed using an Applied BioSystems 4000Qtrap equipped with a turbo spray ion source operated in positive electrospray ionization mode. All aspects of the system operation and data acquisition are controlled using Analyst software by Applied BioSystems.

### 2.4. Online SPE

The Symbiosis system is designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes are eluted directly onto the analytical column, followed by a separation procedure and detection by the mass spectrometer (Fig. 1). This entire procedure is automated and done in series.

For the method herein, the SPE cartridge was first loaded in the left clamp of the ACE for conditioning and equilibration with 1 mL of 100% acetonitrile and water with 3% ammonia respectively (Tables 1 and 2). 30 µL of the pretreated blood was loaded onto the cartridge using water with 3% ammonia as the loading solvent. The cartridge was then washed with 1 mL of water containing 3% ammonia. The cartridge was automatically transferred to the right clamp for elution of the analytes directly onto the analytical column by passing 200 µL of elution solvent (40% acetonitrile/55% water/4% isopropanol/1.1% formic acid) in the peak focusing mode over the cartridge for 3 min. During elution of the cartridge on the right clamp, the sample loop, the left clamp, and the injector were flushed with a series of solvents (Table 3). Immediately following the flushing of the left clamp, the next sample was extracted with



**Fig. 1.** Automated solid phase extraction unit coupled to a triple quadrupole mass spectrometer with (A) autosampler with a sample storage compartment, (B and C) high pressure dispensing pumps, (D) automated cartridge exchanger and (E) triple stage quadrupole mass spectrometer.

**Table 1**  
Autosampler parameters and valve wash sequence between sample injection

Action	Solvent	Volume (mL)	Rate (mL/min)
Condition	100% Acetonitrile	1	5
Equilibrate	Water with 3% ammonia	1	5
Sample application	30 $\mu$ L		
Rinse sample	Water with 3% ammonia	0.5	0.5
Rinse cartridge	Water with 3% ammonia	1	5
Elute	Elution solvent <sup>a,b</sup>	0.3	0.1
Clamp flush # 1	100% Methanol with 3% ammonia	0.5	5
Clamp flush # 2	100% Acetonitrile	0.5	5
Clamp flush # 3	100% Water	0.5	5
Clamp flush # 4	Elution solvent <sup>a</sup>	0.075	5

Cartridge sorbent type: Hysphere MM anion.

<sup>a</sup> Elution solvent: 40% Acetonitrile/55% Water/4% isopropanol/1.1% formic acid.  
<sup>b</sup> Peak focusing with High Pressure Dispenser (HPD).

**Table 2**  
High Pressure Dispenser parameters (HPD-1 and HPD-2)

HPD-1 Port SSM 1A	100% Acetonitrile
HPD-1 Port SSM 1F	Water with 3% ammonia
HPD1 Port 3	40% Acetonitrile/60% water/0.1% formic acid
HPD2 Port 1	100% Acetonitrile
HPD2 Port 2	40% Acetonitrile/55% water/4% isopropanol/1.1% formic acid
HPD2 Port 3	Methanol with 3% ammonia
HPD2 Port 4	Deionized water

**Table 3**  
Parameters for automated cartridge exchanger (ACE)

Injection mode	Partial loopfill	
Syringe speed	Normal	
Injection volume	30 $\mu$ L	
Preflush volume	2 $\times$ needle volume	
Valve wash		
Action	Solvent	Volume (mL)
Rinse	Port 1–Rinse 1 <sup>a</sup>	0.7
Rinse	Port 2–Rinse 2 <sup>b</sup>	0.7
Rinse	Port 1–Rinse 1	0.5
Rinse	Port 2–Rinse 2	0.5
Rinse	Port 1–Rinse 1	0.5
Rinse	Port 2–Rinse 2	0.5
Rinse	Port 1–Rinse 1	1.4

<sup>a</sup> Rinse 1: 40% Acetonitrile/60% water/0.1% formic acid.

<sup>b</sup> Rinse 2: 40% Acetonitrile/40% methanol/15% water/5% isopropanol/0.1% formic acid.

**Table 4**  
Chromatographic parameters

Time (min)	Total flow (mL/min)	%A (0.1% formic acid in water)	%B (0.1% formic acid in acetonitrile)
0.01	0.9	100	0
3.01	0.9	100	0
3.05	1.0	98	2
7.35	1.0	10	90
8.00	1.0	10	90
9.15	1.0	100	0
10.00	1.0	100	0

a new cartridge and placed into standby mode for elution. As a result, there was no lag time in the sample sequence. Additionally, the right clamp was flushed following elution of each sample with methanol containing 3% ammonia, 100% acetonitrile, 100% water and elution solvent (Fig. 1).

## 2.5. Liquid chromatography

A binary gradient system used 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient was performed according to the elution program listed in Table 4. The HPLC column was maintained at room temperature.

## 2.6. Mass spectrometry

In this method, the positive ionization mode was used with the parameters listed in Table 5. E, EME, BZE, C, and CE were protonated to produce the following molecular ions: 186, 200, 290, 304 and 318  $m/z$ , respectively. Likewise, the internal standards ( $d_3$ -E,  $d_3$ -EME,  $d_5$ -BZE,  $d_3$ -C,  $d_3$ -CE) produced ions 189, 203, 295, 307 and 321  $m/z$ , respectively. Upon collision dissociation of E, EME, BZE, C, CE,  $d_3$ -E,  $d_3$ -EME,  $d_5$ -BZE,  $d_3$ -C and  $d_3$ -CE, these precursor ions produced characteristic product ions of 168, 182, 168, 182, 196, 171, 185, 168, 185, and 199  $m/z$ , respectively. Multiple reaction monitoring (MRM) was used with two transitions for each analyte and one transition for each internal standard (Fig. 2). A dwell time of 25 ms and an interchannel delay of 10 ms were used. Mass transitions  $m/z$  186  $\rightarrow$  82 (E), 200  $\rightarrow$  82 (EME), 290  $\rightarrow$  105 (BZE), 304  $\rightarrow$  82 (C), and 318  $\rightarrow$  82 (CE) were used as qualifiers. To ensure no significant hydrolysis of cocaine during the extraction procedure and in solution, production of  $d_3$ -BZE was monitored with transition  $m/z$  293  $\rightarrow$  171.3 (Table 5). The formation of  $d_3$ -BZE is the breakdown product from  $d_3$ -C. To prevent any interference from the breakdown product in monitoring BZE,  $d_5$ -BZE was used as the internal standard.

**Table 5**  
Mass spectrometer parameters

SCAN Mode	Turbo Spray			Polarity	Positive		
Resolution	Unit			Scan Type	MRM <sup>a</sup>		
Curtain Gas	Nitrogen			Ionspray Voltage	2100		
Source Temperature	700 °C			Nebulizer Gas	Nitrogen		
Collision Gas	Nitrogen (Medium)			Turbo Gas	Nitrogen		
Analyte	Q1 mass	Q3 mass	Time (ms)	DP (V) <sup>b</sup>	EP (V) <sup>c</sup>	CE (V) <sup>d</sup>	CXP (V) <sup>e</sup>
Ecgognine (MRM1)	186.15	168.10	25	10	41	25	30
Ecgognine (MRM2)	186.15	82.10	25	10	41	47	12
Ecgognine- <i>d</i> <sub>3</sub>	189.17	171.02	25	10	61	25	12
Ecgognine methyl ester (MRM1)	200.18	182.10	25	10	46	25	44
Ecgognine methyl ester (MRM2)	200.18	82.00	25	10	46	39	4
Ecgognine methyl ester- <i>d</i> <sub>3</sub>	203.20	185.10	25	10	51	27	12
Benzoylecgognine (MRM1)	290.14	168.10	25	10	41	31	14
Benzoylecgognine (MRM2)	290.14	105.10	25	10	41	45	18
Benzoylecgognine- <i>d</i> <sub>5</sub>	295.16	168.20	25	10	91	31	4
Cocaine (MRM1)	304.17	182.10	25	10	71	31	14
Cocaine (MRM2)	304.17	82.10	25	10	71	53	14
Cocaine- <i>d</i> <sub>3</sub>	307.18	185.40	25	10	86	35	16
Cocaethylene (MRM1)	318.20	196.10	25	10	66	27	20
Cocaethylene (MRM2)	318.20	82.10	25	10	66	43	4
Cocaethylene- <i>d</i> <sub>3</sub>	321.23	199.10	25	10	56	27	30
Benzoylecgognine- <i>d</i> <sub>3</sub>	293.16	171.30	25	10	46	31	8

<sup>a</sup> Multiple reaction monitoring

<sup>b</sup> Declustering potential.

### <sup>c</sup> Entrance potential.

<sup>d</sup> Collision energy.

<sup>e</sup> Collision exit potential.

### 3. Results and discussion

In developing this method, ten different solid phase extraction materials were evaluated for the retention of all analytes and their respective internal standards. The C8-EC, C18-EC and C18-HD cartridge extracted C, CE, BZE and EME, but did not retain E. The C2, C8 and Cation Exchange SPE material extracted C and CE, but had poor retention of BZE and EME. The CN cartridge was successful in extraction of C, CE and BZE, but weak retention of EME and E. The SH and GP cartridges extracted C, CE, BZE but showed poor resolution for EME and had no retention for E. However, the Hysphere MM anion SPE material extracted all the analytes. Since this procedure requires online elution of the sample from the SPE material onto the analytical column, consideration must be taken for the SPE elution time, SPE flow rate, elution solvent concentration, LC flow rate and mobile phase. A successful method involves elution of all the analytes at the head of the analytical column without any band broadening. In developing this method several elution solvents, combinations of solvents, and SPE flow rates were evaluated. The most successful elution solvent combination was 300  $\mu$ L of elution solvent at a flow rate of 0.1 mL/min (Table 4). To achieve the shortest runtime with the best separation, eleven analytical columns (Nova-Pak C18, Phenomenex C8 and C18, Phenomenex Synergi Polar-RP (C18), Symmetry C8 and C18, Waters Xterra C8 and C18, Waters Xbridge C8 and C18, and Phenomenex Gemini C6-phenyl were evaluated. The Gemini C6-Phenyl and the Xterra C18 provided optimal separation of all the analytes from the lot of eleven columns. However, the Gemini C6-phenyl provided slightly better peak shape for econine and as a result was used for the study.

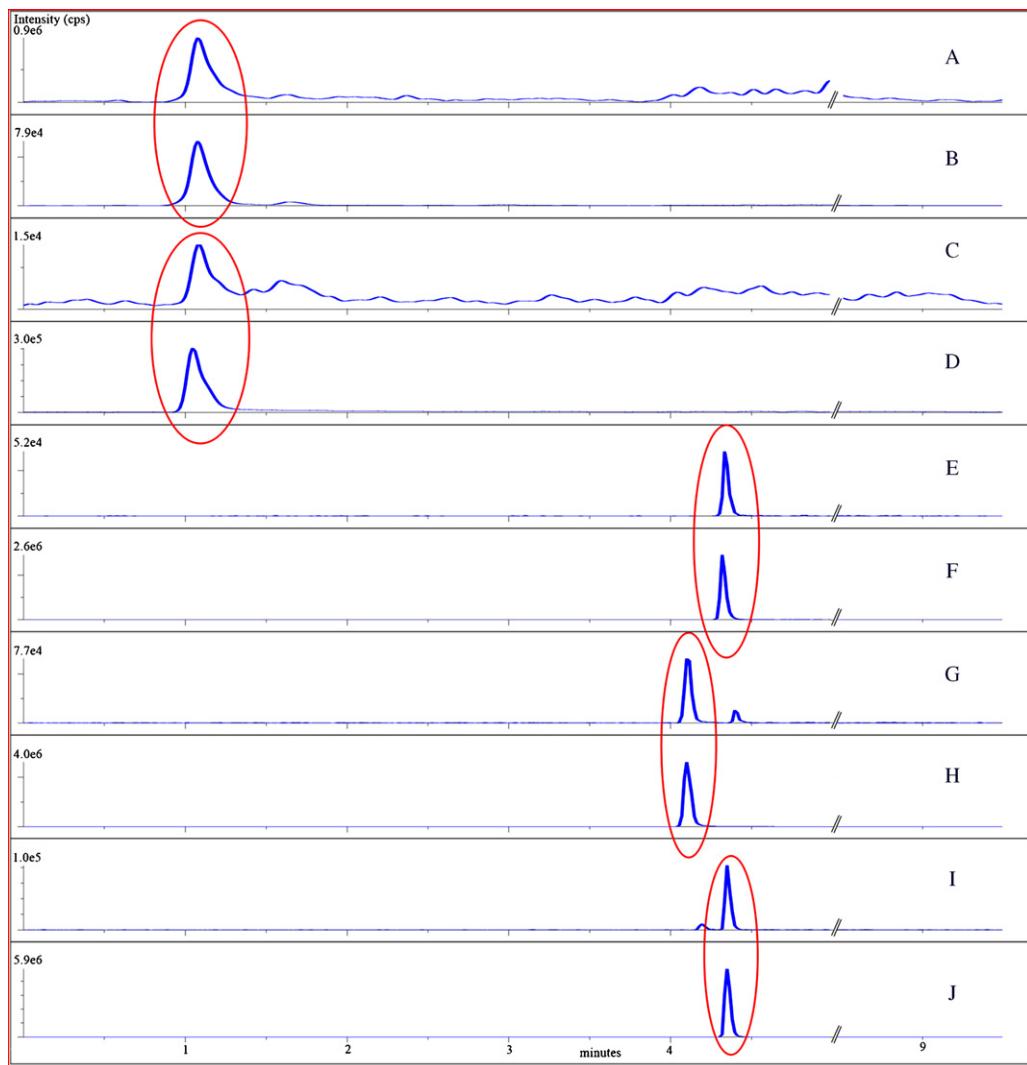
A complete validation was performed following an internal procedure. Interferences were addressed by running blank blood and blood samples spiked with known drugs. Blood samples from ten different sources were analyzed and no endogenous interferences were observed. Also, blood samples spiked with nitrazepam, nordiazepam, alprazolam, flunitrazepam, lorazepam, oxazepam, temazepam, diazepam, propoxyphene, norpropoxyphene, methadone, codeine, phencyclidine, methylenedioxymetha-

mine, methylenedioxymethamphetamine, methylenedioxo-N-ethylamphetamine, methamphetamine, amphetamine, butalbital, amobarbital, phenobarbital, pentobarbital, secobarbital, carisoprodol, lysergic acid diethylamide, oxycodone, zolpidem, 7-aminofunitrazepam, ketamine and imipramine ranging from 1 to 250 ng/mL were analyzed without any significant interferences. Ion suppression was investigated using a post-column tee setup. No significant suppression or enhancement of the signal was observed. Analytes were infused post-column into the LC flow and each extracted blank blood sample injected.

A hydrolysis study of cocaine was studied to determine the percentage of conversion of cocaine to benzoylecgonine over three days by monitoring the ratio of transitions  $(293.16 \rightarrow 168.20)/(295.16 \rightarrow 168.20)$ , while the samples are stored refrigerated at  $12^{\circ}\text{C}$ . The percent conversion was <1% over the three days of study. Storing the blood extract in phosphate buffer at  $12^{\circ}\text{C}$  aids in slowing down this conversion. As a result of the low conversion rate, it is possible to re-extract the sample when necessary without any significant change in the results. This study also indicates that cocaine is not converted to benzoylecgonine during the extraction procedure at an appreciable level, even though strongly alkaline solvents are utilized.

The Symbiosis is comprised of numerous Valco valves, clamps and tubing that provides the potential of analyte carryover. As a result, an extensive study was performed with different solvents and wash cycles to remove any carryover. The optimal conditions to prevent carryover for the working range of the method are listed in Tables 1 and 3.

The method was validated over a range from 4 to 500 ng/mL with an average correlation coefficient >0.99 for all analytes. The standard curves were plots of the ratios of analytes/internal standard responses (peak area) as a function of the analyte concentration. The data were fit to a linear least-squares regression curve with a weighting index of  $1/x$ . Average line equations were determined for all analytes using the equation  $Y = \text{average slope} (\pm 3S.D.) + \text{average intercept} (\pm 3S.D.)$  (Table 6). Although the method was validated in the range 4–500 ng/mL, the linear dynamic ranges for



**Fig. 2.** MRM transitions at of 4 ng/mL in blood for ecgonine, ecgonine methyl ester, benzoylecgonine, cocaine and cocaethylene (A, C, E, G, I) respectively, and MRM transition of 375 ng/mL of  $d_3$ -ecgonine,  $d_3$ -ecgonine methyl ester,  $d_5$ -benzoylecgonine,  $d_3$ -cocaine, and  $d_3$ -cocaethylene (B, D, F, H, J) (Internal standards).

**Table 6**  
Standard curve stability evaluation for cocaine and its metabolites

Curve stability evaluation			
Analyte	Average correlation	Average slope	Average intercept
Ecgonine	>0.998	$0.00126 \pm 0.0003$	$0.0332 \pm 0.0180$
Ecgonine methyl ester	>0.997	$0.00079 \pm 0.00001$	$0.0118 \pm 0.0087$
Benzoylecgonine	>0.998	$0.00216 \pm 0.00039$	$0.0017 \pm 0.0129$
Cocaine	>0.998	$0.00229 \pm 0.00033$	$0.0021 \pm 0.0157$
Cocaethylene	>0.998	$0.00162 \pm 0.0003$	$0.00347 \pm 0.0072$

$n = 5$ . Average line equation  $y = \text{Average slope} (\pm 3\text{S.D.})X + \text{average intercept} (\pm 3\text{S.D.})$ . Data were fit to a linear least-squares regression curve with a weighing index of  $1/X$ .

E, EME, BZE, C and CE were determined to be 50–500 ng/mL, 40–500 ng/mL, 20–500 ng/mL, 10–500 ng/mL, and 15–500 ng/mL respectively (Table 7). The limits of detection for E, EME, BZE, C and CE were determined to be 16 ng/mL, 12 ng/mL, 7 ng/mL, 3 ng/mL and 5 ng/mL respectively. These LODs were statistically determined using the equation  $3.3 \times \text{S.D. } y\text{-intercept}/\text{average slope}$  for 5 calibration curves. Likewise, the limits of quantitation for E, EME, BZE, C, and CE were determined to be 50 ng/mL, 40 ng/mL, 20 ng/mL, 10 ng/mL and 15 ng/mL respectively. These LOQs were statistically determined using the equation  $10 \times \text{S.D. } y\text{-Intercept}/\text{Average Slope}$  for 5 calibration curves. Quality control (QC) samples were analyzed for each analyte in triplicate at 50, 200 and 400 ng/mL. The

**Table 7**  
Critical method performance parameters

Analyte	Limitations		
	Linearity range (ng/mL)	Limit of detection (ng/mL)	Limit of quantitation (ng/mL)
Ecgonine	47–500	16	47
Ecgonine methyl ester	36–500	12	36
Benzoylecgonine	30–500	7	20
Cocaine	8–500	3	8
Cocaethylene	15–500	5	15

LOD was calculated using:  $3.3 \times \text{S.D. of } y\text{-Intercept} / \text{Average Slope}$ . LOQ was calculated using:  $10 \times \text{S.D. of } y\text{-Intercept} / \text{Average Slope}$ .

**Table 8**  
Quality control performance parameters

Analyte	Concentration (ng/mL)	Bias (%)	Intermediate precision (%)
Ecgonine	50	−3.28	7.87
	200	−2.72	8.65
	400	−2.06	6.84
Ecgonine methyl ester	50	−3.78	8.13
	200	−2.89	8.02
	400	−0.41	6.43
Benzoylecgonine	50	−6.96	5.41
	200	−4.04	6.42
	400	−6.16	6.03
Cocaine	50	2.80	3.69
	200	3.65	4.24
	400	0.97	5.69
Cocaethylene	50	2.62	5.34
	200	−0.10	8.55
	400	−2.69	5.84

*n* = 15.

bias and precision were determined using a simple analysis of variance (ANOVA: single factor). The results demonstrated that the bias was <4% for E, EME, C, and CE, while BZE was <7% (Table 8). The precision was determined to be <9% for all analytes at each QC level. As an additional confirmation of identification of an analyte, the ion ratio was calculated and was required to be within 20% of a contemporaneously analyzed calibration standard. Ion ratios for QC at all levels for the entire validation were calculated. The results show that with the exception of one sample, all the ratios adhere to the 20% guideline. Furthermore, with the exception of E and EME, the rest of the analytes (BZE, C, and CE) had ratios that were within 10%.

#### 4. Conclusion

In this study, an efficient method was developed to simultaneously extract cocaine and four of its metabolites from whole blood. This fully automated instrumental method eliminates many of the time consuming, manual sample processing steps used by other SPE-LC-MS methods, thereby minimizing the chance of

errors. This method demonstrates excellent accuracy and precision, and an excellent lower limit of detection. The analytical results demonstrate the feasibility of the technique for high throughput and fully automated analysis, without sacrificing accuracy and precision.

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