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Differentiating Crack Cocaine from Regular Cocaine in Whole Blood Samples in Drugs and Driving Cases.

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Abstract:

In this study, the procedure for analyzing cocainic drugs and metabolites: (anhydroecgnonine, anhydroecgnonine methyl ester, benzoylecgonine, cocaine, cocaethylene, ecgnonine ethyl ester, and ecgnonine methyl ester) in whole blood samples obtained from drugs and driving cases using a mixed mode solid phase extraction (SPE) is described. This extraction and analysis procedure allows forensic analysts to differentiate between drivers who have used “crack cocaine” against those using regular cocaine. Samples of whole blood (containing deuterated internal standards) were diluted with an aqueous phosphate buffer (pH 6). Each sample was applied to a conditioned SPE column. The filtrate was collected and adjusted to pH 2. The sorbent was rinsed with deionized (DI) water, aqueous hydrochloric acid and methanol. After drying, cocaine, cocathylene, benzoylecgnonine, and anhydroecgnonine methyl ester were eluted from the SPE column with 3 mL of an elution solvent consisting of methylene chloride/ isopropanol/ ammonium hydroxide. The original filtrate was

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applied to a second conditioned SPE column and washed with DI water, aqueous hydrochloric acid and methanol. After drying the SPE sorbent, the anhydroecgnonine was eluted with methanol containing 4% ammonium hydroxide (3 mL). The eluates were combined and evaporated to dryness, and the residue was dissolved in mobile phase for analysis by LC-MS/MS in positive multiple reaction monitoring mode (MRM) Chromatography was performed in gradient mode employing a C_{18} column and a mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. The total run time for each analysis was under five minutes.

The limits of quantitation/ detection for this method were determined to be 0.5 ng/mL and 1.0 ng/mL respectively. The method was found to be linear from 1.0 ng/ mL to 100.0 ng/mL (r^2>0.995). The recoveries of the noted cocaine type drugs found to be greater than 90%. This method was applied to twenty positive cocaine completed drugs and driving cases, it was found that anhydroecgnonine concentrations ranged 0 to 23 ng/ mL, anhydroecgonine methyl ester concentrations ranged from 0 to 66 ng/ mL, while cocaine was found to range from 25 to 250 ng/ mL , cocaethylene ranged from 0 to 80 ng/ mL, benzoylecgnonine concentrations were found to be in the range 90 to 790 ng/ mL, ecgonine methyl ester concentrations ranged from 40 to 500 ng/ mL and ecgonine ethyl ester concentrations were found to range 0 to 180 ng/ mL.

Keyword: Cocaine, Crack, Blood, SPE, Chromatography

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3 48 **Introduction**
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6 49 Cocaine is a tropane type[1] compound found in nature in the coca bush (a member of the genus
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8 50 Erythroxylon). It has been used since antiquity by the indigenous peoples of South America for
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11 51 religious purposes. In 1859, the pure compound was isolated from coca leaves by Albert
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13 52 Niemann [2], although its synthesis and structural elucidation was first performed by Richard
14
15 53 Willstatter in 1898 [3]. Cocaine possesses the properties of a strong nervous system stimulant [4]. Its
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17 54 effects can last from fifteen to sixty minutes. This is dependent on the amount of the intake
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20 55 dosage and the route of administration [5] Cocaine can be in the form of fine white powder, bitter
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22 56 to the taste. When inhaled or injected, it causes a numbing effect. "Crack" cocaine is a
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25 57 smokeable form of cocaine made into small "rocks" by processing cocaine with sodium
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27 58 bicarbonate (baking soda) and water.
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30 59 Cocaine increases alertness, feelings of well-being and euphoria, energy and motor
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32 60 activity, feelings of competence and sexuality. Anxiety, paranoia and restlessness can also occur,
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35 61 especially during the comedown. With excessive dosage, tremors, convulsions and increased
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37 62 body temperature are observed [4].Severe cardiac adverse events, particularly sudden cardiac
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39 63 death, become a serious risk at high doses due to cocaine's blocking effect on cardiac sodium
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42 64 channels [6].
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45 65 Cocaine is known to undergo spontaneous chemical hydrolysis to form benzoylecgnonine
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47 66 in biological samples [6]. This compound is pharmacologically inactive but important as screening
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50 67 by immunoassay targets this compound. Metabolism of cocaine proceeds via enzymatic
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52 68 hydrolysis to form ecgonine methyl ester [7-9]. Ecgnonine ethyl ester is formed from the
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54 69 breakdown of cocaethylene in the body, which is formed *in vivo* when cocaine and ethanol are
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57 70 administered together [10]. When "crack" is administered by heating the material, the pyrolytic
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71 compounds anhydroecgonine and anhydroecgonine methyl ester have been reported in the
72 literature of forensic science to have been found in human systems. [11-13]. Other metabolites of
73 cocaine include norcocaine, p-hydroxycocaine, m-hydroxycocaine, p-hydroxybenzoylecgonine,
74 and n-hydroxybenzoylecgonine[14]. These compounds were not part of this study.

75 This use of analyzing blood for AEME and AE is useful for analysts wishing to
76 differentiate between regular cocaine users and those using "crack". One of the issues with using
77 gas chromatography-mass spectrometry (GC-MS) for the analysis of cocaine/"crack" metabolites
78 is that it has been reported that AEME may be formed at the injection port of the GC in the GC-
79 MS unit [15-17]. In this study, analysis was performed by use of LC-MS/MS where the injection
80 port is maintained at room temperature thus eliminating any high temperature conversion of
81 cocaine to AEME.

82 Currently, screening for these compounds is often accomplished by immunoassay
83 followed by GC/MS for confirmation and/or quantitation. However, due the availability of
84 LC/MS/MS, many frequently used procedures including cocainic type drugs are being developed
85 away from the traditional GC-MS onto this instrument. LC/MS/MS is a complementary
86 technique to GC/MS and the very compounds that are challenging to analyze by GC/MS, i.e.
87 polar, amines, and semi-volatile compounds, are ideal candidates for LC/MS/MS analysis.
88 Furthermore, sample preparation is often greatly simplified as the final step of sample
89 preparation i.e. derivatization can be omitted and the sample dissolved in the mobile phase. In
90 GC-MS analysis derivatization is required for the analysis of benzoylecgonine, and has typically
91 been performed using silyl reagents such as BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide)
92 or PFPA (pentafluoropropionic anahydride). [11,18-19]

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Previous methods of analysis for cocaine have included both the previously noted GC-MS methods and LC-MS/MS procedures [20-25], extraction of the drugs has been performed by SPE [26-28] and liquid-liquid extraction (LLE) [29-30]. Our procedure applies both the technologies of SPE and LC-MS/MS to produce a robust, efficient method that maximizes the information for forensic toxicologists in a timely fashion regarding cocaine related drugs and driving cases, especially those where "crack" cocaine has been used. This is demonstrated by the presented results of twenty completed cases where cocaine, cocaethylene, benzoylecgnonine, ecgonine ethyl ester, and ecgonine methyl ester were previously quantified but not anhydroecgnonine and anhydroecgnonine methyl ester.

Of interest to forensic toxicologists involved in drugs and driving cases is whether the offender has been using regular cocaine or "crack" cocaine, as some authorities place more emphasis on prosecuting "crack" cocaine users due to its stigma. By analyzing blood samples for the pyrolysis components of cocaine (anhydroecgonine (AE)/anhydroecgonine methyl ester (AEME) this differentiation can be made. In this procedure, one aliquot (1 mL) of the whole blood sample is taken and buffered with pH 6 phosphate buffer and applied to a pre-conditioned mixed mode (C8/Strong cation exchanger) SPE cartridge and allowed to pass through the sorbent. Conventially, the filtrate is aspirated to waste[31], but in this methodology the filtrate is collected and adjusted to pH 2 with glacial acetic acid for re-extraction on a separate mixed mode SPE containing the same chemistry as before. By using the second extraction AE can be isolated from the matrix, thus permitting analysts to combine the first eluate containing AEME, benzoylecgnonine, cocaine, cocaethylene, ecgonine ethyl ester, and ecgnonine methyl ester with the second eluate containing AE to provide a broad spectrum of cocainic type drugs for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This group of compounds

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116 also allows the analyst to provide information not only regarding the use of “crack” cocaine over
117 regular cocaine but also whether ethanol and cocaine (by confirmation of cocaethylene) have
118 been involved.

119 This developed method should be of interest to forensic toxicologists involved in drugs
120 and driving cases is whether the offender has been using regular cocaine or “crack” cocaine, as
121 some authorities place more emphasis on prosecuting “crack” cocaine users due to its stigma. By
122 analyzing blood samples for the pyrolysis components of cocaine i.e. anhydroecgonine
123 (AE)/anhydroecgonine methyl ester (AEME)) this differentation can be made. In this procedure,
124 one aliquot (1 mL) of the whole blood sample is taken and buffered with pH 6 phosphate buffer
125 and applied to a pre-conditioned mixed mode (C8/Strong cation exchanger) SPE cartridge and
126 allowed to pass through the sorbent. At this pH, anhydroecgnonine is not retained by the SPE
127 sorbent as the other compounds are and must be collected as a filtrate. Conventially, the filtrate is
128 aspirated to waste[1], but in this methodology the filtrate is collected and adjusted to pH 2 with
129 glacial acetic acid for re-extraction on a separate mixed mode SPE containing the same
130 chemistry as before. At pH 2, anhydroecgonine is made a neutral moiety, thus making it
131 amenable for sorbtion on the SPE sorbent. By using the second extraction AE can be isolated
132 from the matrix, thus permitting analysts to combine the first eluate containing AEME,
133 benzoylecgnonine, cocaine, cocaethylene, ecgonine ethyl ester, and ecgnonine methyl ester with
134 the second eluate containing AE to provide a broad spectrum of cocainic type drugs for analysis
135 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This group of compounds
136 also allows the analyst to provide information not only regarding the use of “crack” cocaine over
137 regular cocaine but also whether ethanol and cocaine (by confirmation of cocaethylene) have
138 been involved.

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This method was developed and validated according to the policies and procedures employed at the Massachusetts State Police Crime Laboratory. This procedure was performed manually, but as technology moves on, there is no reason why automation cannot be used in a programmed instrument. This procedure employs SPE and LC-MS/MS for looking at AE/AEME/COC/BE/BZE in whole blood. To date the authors have not seen any reports for these analytes using the configuration for the analysis in drugs/driving cases

Experimental

Chemicals and reagents

Anhydroecgnonine (AE), anhydroecgnonine methyl ester (AEME), benzoylecgnonine (BE), cocaine (COC), and cocaethylene (CE) were obtained as 1 mg/ mL acetonitrile solutions from Lipomed (Cambridge MA, USA). The tri -deuterated solutions of benzoylecgnonine (BE-d_3),cocaethylene (CE-d_3) and cocaine (COC-d_3) were obtained as 0.1 mg/ acetonitrile solutions from the same supplier. Ecgnonine ethyl ester (EEE), ecgonine methyl ester (EME) were obtained as 1 mg/ mL acetonitrile solutions from Cerilliant (Round Rock TX USA). Ecgonine methyl ester-d_3 (EME-d_3) was obtained as a 0.1 mg/ mL acetonitrile solution from the same supplier.

Acetonitrile, acetic acid (glacial), concentrated ammonium hydroxide solution (32% by volume), formic acid, hydrochloric acid (37% by volume), isopropanol, methanol and methylene chloride were obtained from Fisher Scientific (Pittsburgh PA, USA). The SPE columns (CSDAU206) were obtained from UCT Inc., (Bristol PA, USA). These SPE cartridges contained 200 mg of sorbent in 6 mL SPE tubes. Deionized (DI) water was laboratory grade and it was

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generated in the Massachusetts State Police Crime Laboratory (MSPCL). All chemicals were of ACS grade.

Hydrochloric acid was prepared as 0.1 M aqueous solution by adding 8.4 mL of the stock solution (37% by volume) to 500 mL, mixing, diluting to 1 L with DI water and mixing well. Formic acid was prepared as a 0.1% (v/v) solution by the addition of 1 mL of the acid to 900 mL of DI water and diluting to 1 L (mobile phase solvent A). Acetonitrile containing 0.1% formic acid (v/v) was prepared by adding 1 ml of formic acid to 900 mL of acetonitrile and diluting to 1 L (mobile phase solvent B). Phosphate buffer (pH 6, 0.1 M) was purchased from Fisher Scientific as a ready to use solution.

Chromatographic analysis.

Analysis was performed using an API 3200 Q-Trap instrument supplied by Applied Biosystems (Foster City, CA). The chromatographic system consisted of a Shimadzu CBM 20 A controller, two Shimadzu LC 20 AD pumps including degasser, a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO AC oven (set at 10 ˚C). The instrument was fitted with a Unison-C_{18} column: **50mm x 2 mm, 5 µm particle size** from Silvertone Sciences (Philadelphia PA, USA), and was attached to a Unison-C_{18} guard column: 5 mm x 2mm, **5µm particle size,** which was obtained from the same supplier. The liquid chromatography column oven was maintained at 40 °C throughout the analyses. The injection volume was 10 µl. The mobile phase consisted of solvent A: DI water containing 0.1% formic acid and solvent B: acetonitrile containing 0.1% formic acid, delivered at a flow rate of 0.5 mL/minute. The LC gradient program is presented in Table 1. The retention times of the individual cocainic drugs and metabolites were found to AE (0.71 minutes), AEME (0.98 minutes), BE (2.38

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3 183 minutes), COC (2.60 minutes), CEC (2.72 minutes), EEE (0.83 minutes), and
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6 184 EME (0.65 minutes).
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8 185 **The analysis of the samples was performed under laws pertaining to the**
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11 186 **Commonwealth of Massachusetts regarding testing of whole blood samples**
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13 187 **of suspects operating motor vehicles under the influence of drugs (OUI). The**
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15 188 **samples were submitted to the laboratory on behalf of the Office of the**
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18 189 **District Attorney by Massachusetts State Police**.
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27 193 Tandem Mass Spectrometer
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30 194 The mass spectrometry was performed on an API 3200 Q-Trap using positive multiple
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32 195 reaction monitoring (MRM). The mass spectrometer conditions for each of the cocaine type
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34 196 drugs are shown in Table 2. Tandem mass spectrometry was performed under the following
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37 197 conditions: curtain gas setting= 15, collision gas setting= medium, ion spray voltage setting=
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39 198 5000V, temperature setting= 650 °C, ion source gas #1 setting= 50, ion source gas #2 setting=
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42 199 50. Tandem mass spectrometer conditions are shown in **Table 2**. The analytical data was
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44 200 collected using Analyst Software Version 1.5.2 supplied by Applied Biosystems. 50. Data was
45
46 201 collected using Analyst Software Version 1.5 (Applied Biosystems).
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49 202 Positive confirmation of the compounds was based upon peak retention time and the
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51 203 ratios of the MRM. Two MRM's were employed, the major one was used for quantification, and
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53 204 the second was used as a qualifier. The retention time of the peaks had to be within 0.2 minutes
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56 205 of the lowest standard, and ratio of the two transitions had to be less than 25%.
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207 Sample Preparation for analysis

208 A 1000ng/ mL working solution of the cocaine type drugs consisting of AE, AEME, BE,
209 COC, CEC, EEE, and EME was prepared by diluting 10 μL of the corresponding
210 1 mg/ mL solution to 10 mL with acetonitrile in a volumetric flask. The
211 resulting solution was transferred to a glass screw top vial. A 1000 ng/ mL
212 working solution of the deuterated internal standards was prepared by diluting
213 100 μL to 10 mL with acetonitrile in a volumetric flask. This solution was
214 transferred to a glass screw top vial. Both working solutions were stored in a
215 refrigerator (4°C) until ready to use.

216 Calibrators and controls

217 Calibrators were prepared by the addition of 1.0 μL, 5.0 μL, 10.0 μL 25 .0 μL, 50.0 μL
218 and 100.0 μL of cocainic drugs and metabolite solution consisting of AE, AEME, BE, COC,
219 CEC, EEE, and EME into 1 mL samples of drug free whole blood samples. To these samples
220 50 μL of the internal standard solution was added. Control samples were prepared by the
221 addition of 4 μL and 80 μL of the cocainic drugs and metabolite solution to 1 mL of drug free
222 whole blood samples in addition to 50 μL of the internal standard solution.

223 Test samples

224 Test samples were chosen from cocaine positive blood samples that had been previously
225 analyzed and reported for the concentrations of COC, CEC, and BE, EEE, and EME but not for
226 AE and AEME. These samples were prepared by adding 50 μL of internal standard solution to 1
227 mL aliquots of whole blood sample.

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3 228 All determinations were performed in duplicate. A negative control sample was prepared
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6 229 by the addition of 50 μL the internal standard to 1 mL sample of drug free whole blood.
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8 230 Calibrators, control samples, and test samples were treated in an identical mode with regard to
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10 231 sample extraction i.e. after buffering with 3 mL of pH 6 phosphate buffer of concentration 0.1M,
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13 232 the samples were vortex mixed for approximately 1 minute then centrifuged at 3000 rpm for 10
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15 233 minutes. The supernatant liquid was applied to a pre-conditioned SPE cartridge.
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20 235 Solid Phase Extraction
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22 236 Solid phase extraction columns were conditioned by the sequential addition of: 1 x 3 mL
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24 237 of methanol, 1 x 3 mL of DI water, and 1 x 1 mL of pH 6 phosphate buffer, of 0.1 M
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27 238 concentration. Each liquid was allowed to percolate through the sorbent using gravity without
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29 239 allowing the sorbent to dry out in between steps.
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32 240 Following the passage of the methanol, DI water and pH 6 phosphate buffer of
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34 241 concentration 0.1 M through the SPE columns, each diluted sample i.e. calibrator, control, and
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36 242 test sample was loaded on to an individually marked SPE tube, and allowed to pass through the
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39 243 sorbent using gravitational flow. The filtrate (**F**) was collected in 12mm x 75 mm glass tubes for
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41 244 further extraction.
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43 245 The SPE columns were then washed with: 1 x 3 mL of DI water, 1 x 1 mL of 0.1 M
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46 246 hydrochloric acid, and 1 x 3 mL of methanol, respectively. The SPE columns were then dried by
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48 247 applying a vacuum to the SPE manifold at 15 inches of mercury pressure via an electric vacuum
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51 248 pump.
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53 249 The analytes were eluted from the SPE columns by the addition of 1 x 3 mL of a solution
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55 250 consisting of methylene chloride-isopropanol-ammonium hydroxide in the ratios 78:20:2 v/v.
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This solution was prepared daily by adding 2 mL of concentrated ammonium hydroxide solution to 20 mL of isopropanol and mixing well. To this solution was added 78 mL of methylene chloride, and the resultant solution was transferred to a clean screw top bottle for use. The elution solvent was allowed to flow through the SPE sorbent with the aid of gravity and collected in separate glass tubes of dimensions 75 mm x 12 mm.

Extraction of Anhydroecgnonine

The collected filtrate (**F**) from the initial SPE extraction was adjusted to pH 2 with the aid of 100 µL of glacial acetic acid. This solution was further vortex mixed for approximately 1 minute before being centrifuged for 10 minutes at 3000 rpm. The supernatant was applied to a second CSDAU206 SPE column which had been conditioned with 3 mL of methanol, 3 mL DI water and 1 mL of 0.1 M aqueous hydrochloric acid, respectively. After the samples of the filtrate **(F)** had passed through the CSDAU206 SPE cartridges, the SPE cartridges were washed with 3 mL of DI water, 3 mL 0.1 M aqueous hydrochloric acid, and 3 mL of methanol. Each SPE column was dried for 10 minutes and eluted with 3 mL of a solution of 4% ammonium hydroxide in methanol.

Both eluates (methylene chloride-isopropanol-ammonium hydroxide (containing AEME, BE, COC, and CE, EME, and EEE) and ammonium hydroxide-methanol (containing AE)) were combined, mixed and evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was dissolved in 100 µL of a solution containing 95% of mobile phase component (MPA) and 5% of mobile phase component (MPB). This solution was transferred to an autosampler vial containing a low volume insert of volume 100 µL for analysis by LC-MS/MS.

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6 275 Matrix Effects
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8 276 Studies into the matrix effects were performed according to a previously published
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11 277 procedure [32]. In this part of the study, aliquots of the noted cocainic drugs and metabolites
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13 278 (covering the linear range) were introduced into 100 µL of a solution containing 95% of mobile
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15 279 phase solvent MPA and 5% mobile phase solvent MPB. Each of the solutions were evaporated
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18 280 to the mobile phase and analyzed by LC-MS/MS (**A**). Concurrently, a set of whole blood
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20 281 samples were subjected to the SPE process noted, after elution of the SPE columns, the elution
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22 282 solvent was fortified with the noted cocaine type drugs and evaporated to dryness before being
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25 283 dissolved in 100 µL of 95% MPA and 5% MPB. A second set of whole blood samples were
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27 284 fortified with the noted cocaine type drugs and processed via the SPE method. After elution and
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30 285 evaporation to dryness, 100 µL of mobile phase solution was added to dissolve the residue (**C**).
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32 286 The data (peak areas) for A, B, and C were collected by Analyst 1.5.2. By comparing the peak
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34 287 areas of B with those of A an assessment of matrix effects was made. The comparison of peak
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37 288 areas for C with B provided data for the recoveries. To evaluate these procedure samples of drug
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39 289 free whole blood were obtained from 5 individual sources for use.
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44 291 Ion Suppression
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46 292 A 50 ng/ mL solution of cocaine type drugs was infused into the tandem mass
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49 293 spectrometer using the on board syringe pump (controlled by Analyst 1.5.2 software) via a
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51 294 Hamilton syringe (model# 1001TLL, 1 ml volume) (supplied by Fisher Scientific) at a flowrate
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53 295 of 5 µL/ minute. At the same time as the solution of cocainic drugs and metabolites was flowing
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56 296 into the mass spectrometer, a 10 µL aliquot of the SPE extracted whole blood matrix . These
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297 were samples of whole blood confirmed to contain no drug material. The samples were obtained
298 from 5 individual sources. The extracts were injected using the autosampler syringe on the
299 Shimadzu liquid chromatograph. The liquid chromatograph and mass spectrometer were
300 arranged so that samples from the liquid chromatograph were mixed into the flow of cocainic
301 drugs and metabolites via a 3 port T section before the total flow entered the mass spectrometer.
302 Any suppression effects on the cocaine type drugs could be monitored at the MRM's for the
303 noted drugs.
304
305 Selectivity
306 In analyzing samples of whole blood extracts via SPE and LC-MS/MS it is essential to
307 ensure that the interfering effects of other drug compounds can be eliminated. In this procedure,
308 samples of whole blood extracts were spiked with a cocktail of drugs at a concentration
309 equivalent to of 100 ng/ mL of whole blood sample: (bupropion, lidocaine, methadone,
310 amitriptyline, nortriptyline, thioridazine, trazodone, mesoridazine, meperidine, diphenhydramine,
311 phenyltoloxamine, imipramine, desipramine, benztropine, trimethoprim, diltiazem, haloperidol,
312 strychnine, morphine, codeine, 6-acetylmorphine, oxycodone, oxymorphone, hydrocodone,
313 noroxycodone, hydromorphone, diazepam, nordiazepam, oxazepam, temazepam, alprazolam,
314 α-hydroxyalprazolam, lorazepam, triazolam, α-hydroxytriazolam, flunitrazepam, 7 amino-
315 flunitrazepam, chlordiazepoxide, midazolam, α-hydroxymidazolam, flurazepam, desalkyl-
316 flurazepam, clonazepam, 7 amino-clonazepam) and extracted according to the SPE method. It
317 was observed that the interfering effect of these compounds was not found to be significant.
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319 **Results and Discussion**

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6 321 It was found that the mean recovery of cocaine type drugs: AE, AEME, BE, COC,
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8 322 CEC, EEE, and EME from drug free blood samples was determined to be 91 %. (range: 89%-
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10 323 92 % i.e. (AE (89%), AEME (90%), BE (91%), COC(92%), CEC (92%), EEE, (91%), and
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12 324 EME (90%), respectively). This is an excellent indicator for the efficiency of the extraction
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14 325 procedure of cocainic type drugs and metabolites using whole blood as a matrix.
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16 326 To assess the performance of the procedure, calibration curves were constructed twice daily over
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18 327 five consecutive days using the spiked controls, from this data intra-day and inter- day values
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20 328 were obtained.
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26 331 Imprecision of Analysis
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28 332 The results of the analysis of the spiked control samples of whole blood: 4 ng/ mL, 80
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30 333 ng/ mL, respectively are shown in Table 3. Analysis of the control samples was performed at the
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32 334 same time as the calibration curves were constructed i.e. over a period of five days. Control
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34 335 samples were prepared by adding the cocaine type drug solution containing: AE, AEME, BE,
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36 336 COC, CEC, EEE, and EME) to 1 mL of drug free whole blood samples and treating as per
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38 337 the test samples.
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40 338 Intra-day and inter-day variation for the analysis of the cocainic drugs and metabolites:
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42 339 AE, AEME, BE, COC, CEC, EEE, and EME was found to be less than 8% and less than
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44 340 12%, respectively. This method was found to be linear (r^2> 0.995) over the dynamic range 1.0
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46 341 ng/ mL to 100 ng/ mL.
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343 LOD/LOQ

344 The limit of detection (LOD) of a particular method can be defined as the level at which
345 the signal to noise ratio for the particular analyte is greater than or equal than 3:1. The limit
346 quantification (LOQ) for the method is the level at which the signal to noise ratio for a particular
347 analyte is greater than or equal to 10:1. In this study, LOD values were determined empirically
348 by analyzing extracted samples of drug free whole blood fortified with cocainic drugs and
349 metabolites by LC-MS/MS according to the SPE method. This was performed until the lowest
350 level at which each of the respective analytes just failed the signal to noise ratio of 3:1. This was
351 observed to be 0.5 ng/ mg. In terms of LOQ, samples of drug whole blood samples were spiked
352 with the cocaine type drugs at concentrations below 10 ng/ mL and extracted according to the
353 SPE procedure until the analytes could just failed a signal to noise ratio of 10:1; this value was
354 found to be 1.0 ng/ mL. Representative chromatograms at LOQ and genuine blood samples are
355 shown in Figures1-3.

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357 Solid Phase Extraction

358 In this procedure, dilution of the sample of whole blood with an aqueous pH 6 buffer permits
359 both efficient flow and optimal sorbing of the drugs onto the SPE sorbent. In employing a mixed
360 mode SPE. This procedure employs hydrophobic C_8 and strong cation exchange chemistries, the
361 sample can be cleaned up via aqueous hydrochloric acid and methanol washes leaving the drugs
362 in a much cleaner state than when they were originally applied to the SPE column. This effect is
363 noted in the low matrix effects and ion suppression values. This initial procedure permits several
364 of the cocainic drugs and metabolites i.e. anhydroecgonine methyl ester, benzoylecgnonine,

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3 365 cocaethylene, ecgnonine ethyl ester, and ecgonine methyl ester to be efficiently extracted from
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6 366 whole blood samples.
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8 367 In this procedure, anhydroecgonine is extracted in a second step from the same sample of
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11 368 blood. By lowering the pH of the sample with the aid of glacial acetic acid, the molecule
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13 369 becomes protonated and is extracted by both hydrophobic and ion exchange mechanisms. This is
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15 370 demonstrated by the use of aqueous hydrochloric acid to condition the SPE sorbent, thus
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18 371 producing a protonated phase for efficient sorbing of the anhydroecgonine, and the employment
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20 372 of a stronger basic elution solvent (4% ammonium hydroxide in methanol) to overcome the
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23 373 strong cation interactions of the SPE sorbent. The combined eluates are then
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25 374 evaporated/dissolved in mobile phase to produce a single solution for analysis of seven
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27 375 compounds.
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32 377 Tandem Mass Spectrometry
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34 378 In this methodology, LC-MS/MS has been successfully applied to the extraction and
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37 379 analysis of cocainic drugs and metabolites type drugs rather than GC-MS where a derivatization
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39 380 procedure i.e. reaction with a silyl reagent such as BSTFA or an acyl reagent such as
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42 381 pentafluoropropionic anhydride where derivatization , evaporation, and re-constitution in a
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44 382 volatile solvent is required not only to quantify, but also confirm the identity of the cocaine
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46 383 metabolite i.e. benzoylecgnonine. By employing LC –MS/MS with specific MRM's, the
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49 384 individual cocaine type drugs can be targeted, confirmed, and quantified in whole blood samples
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51 385 without the use of derivatization. This procedure coupled with a quick LC method offers
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53 386 analysts the ability of determine concentrations of the drug within a short turnaround time. This
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3 387 method offers the toxicological analyst in a forensic setting the ability to provide information
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6 388 regarding the use of "crack" cocaine rather than regular cocaine in drug related driving cases.
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11 390 Matrix Effects
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13 391 The study found that the matrix effects for the procedure were found to be less than 6% for all
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15 392 the drugs studied.
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22 395 Ion Suppression
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25 396 Ion suppression studies determined that suppression of monitored ions was less than 6 %.
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29 398 Analysis of Genuine Samples.
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32 399 **Table 4** presents the results of 20 drugs and driving cases that had previously been analyzed for
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34 400 cocaine type drugs. These samples had not been previously analyzed for AE or AEME. This
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37 401 table shows that offenders who had used regular cocaine can be differentiated from those who
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39 402 have used "crack" cocaine e.g. samples of whole blood from cases # 1, 3, and 5, in these cases
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41 403 no AE or AEME was observed. Cases were ethanol and cocaine concomitantly can be seen in
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44 404 samples of whole blood from cases such as #3, 5, and 10, in these the presence of cocaethylene
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46 405 was confirmed and quantified. In several examples i.e. samples of whole blood obtained from
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48 406 cases #14, 15, 17, 18, and 20 , cocaethylene was found along with AE and AEME in the blood
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51 407 samples, suggesting that ethanol and "crack" cocaine had been used in these cases.
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53 408 These cases were previously analyzed for cocaine type compounds but not for their
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55 409 AE/AEME concentrations.
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Conclusion

This study shows that extraction and analysis of cocainic drugs and metabolites especially androecgnonine, which is a compound pyrolytically derived from cocaine, can be used by forensic toxicological analysts involved in drugs and driving cases. The use of a single sample of the whole blood samples eliminates the need for the analyses to be performed on two separate aliquots of the case sample. By employing liquid chromatography coupled to tandem mass spectrometry reduces laboratory time spent on derivatization such as with benzoylecgnonine. In this procedure seven cocainic drugs and metabolites can be efficiently extracted and analyzed, thus permitting forensic toxicologists the ability of offer a comprehensive interpretation to submitting agencies especially with reference to whether cocaine was used in the presence of ethanol due to the determination of the concentration of cocathylene in the blood samples. This procedure does also allow forensic toxicologists to gain information with regard to whether regular cocaine or "crack" cocaine was the drug of administration shown by the concentrations of anhydroecgonine in the whole blood sample.

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Time/ minutes	%B (Acetonitrile containing 0.1% formic acid)
0	5
1.0	5
4.0	90
5.0	5
5.1	stop

Table 1. Gradient Program for LC

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Table 2: Tandem Mass Spectrometry Conditions

Compound	Q1	Q3	DP/ volts	EP /volts	CEP /volts	CE/ volts	CXP /volts
AE (1)	168.1	150.2	12.5	10	13.7	35	20
AE(2)	168.1	136.1	12.5	10	13.7	35	15
AEME(1)	182.1	91.1	12.5	10	14.4	50	15
AEME(2)	182.1	122.1	12.5	10	14.4	20	15
COC (1)	304.2	182.0	12.5	10	17.8	20	15
COC (2)	304.2	105.1	12.5	10	17.8	50	15
COC-D3(1)	307.1	185.1	12.5	10	17.4	20	15
COC-D3 (2)	307.1	105.1	12.5	10	17.4	50	15
BE(1)	290.1	168.1	12.5	10	17.3	50	15
BE(2)	290.1	105.1	12.5	10	17.3	50	15
BE-D3(1)	293.3	171.1	12.5	10	17.4	20	15
BE-D3(2)	293.3	85.0	12.5	10	17.4	35	15
CE (1)	318.2	196.4	12.5	10	18.2	20	15
CE (2)	318.2	82.2	12.5	10	18.2	50	15
EEE	213.3	82.1	12.5	10	14.8	35	15
EEE	213.3	168.1	12.5	10	14.8	29	15
EME	200.1	185.2	12.5	10	14.3	20	15
EME	318.2	82.2	12.5	10	14.3	35	15
EME-D3	203.3	182.1	12.5	10	14.4	20	15
EME-D3	203.3	82.1	12.5	10	14.4	50	15

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Note: Q1= Precursor ion; Q3= Product ion; DP= Declustering Potential; EP=Exit Potential; CE=Collision Energy; CXP=Collision Exit Potential

Note: AE= Anhydroecgonine; AEME=Anhydroecgnonine methyl ester; COC=Cocaine; BE=Benzoylecgnonine; CE=Cocaethylene; EME= Ecgnonine methyl ester; EEE= Ecgnonine ethyl ester

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Table 3. Imprecison results of analysis:

Recovery data results for control whole blood samples

Compound	4.0 ng/mL (±)	80 ng/ mL (±)
AE	3.9±0.6	78±9
AEME	3.8±0.5	83±11
BE	4.1±0.3	78±6
COC	4.0±0.3	81±5
CE	3.9±0.3	82±5
EEE	4.1±0.4	78±5
EME	3.9±0.5	83±10

Note: AE= Anhydroecgonine; AEME=Anhydroecgnonine methyl ester; COC=Cocaine; BE=Benzoylecgnonine; CE=Cocaethylene; EME= Ecgnonine methyl ester; EEE= Ecgnonine ethyl ester

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Table 4. Results of 20 case samples analyzed via SPE procedure

Case	AE (ng/mL)	AEME (ng/mL)	COC (ng/ mL)	BE (ng/mL)	CE (ng/mL)	EME (ng/mL)	EEE (ng/mL)
1	n/d	n/d	80	100	n/d	81	n/d
2	23	66	105	560	n/d	390	n/d
3	n/d	n/d	150	850	55	480	190
4	20	50	75	220	n/d	110	n/d
5	n/d	n/d	230	620	15	350	n/d
6	<5	26	75	230	n/d	190	n/d
7	n/d	n/d	90	190	45	110	90
8	<5	48	250	520	n/d	380	n/d
9	n/d	n/d	11	150	n/d	90	n/d
10	n/d	n/d	110	650	80	500	160
11	<5	65	190	790	n/d	400	n/d
12	n/d	n/d	10	90	n/d	40	nd
13	n/d	n/d	230	550	n/d	280	n/d
14	5	27	300	650	60	380	n/d
15	11	35	380	770	90	320	180
16	n/d	n/d	25	220	n/d	180	n/d
17	18	55	180	370	80	200	180
18	9	45	100	250	40	180	110
19	n/d	n/d	60	150	n/d	90	n/d
20	16	60	200	450	75	230	110

Note: AE= Anhydroecgonine; AEME=Anhydroecgnonine methyl ester; COC=Cocaine; BE=Benzoylecgnonine; CE=Cocaethylene; EME= Ecgnonine methyl ester; EEE= Ecgnonine ethyl ester,n/d=not detected.

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Figure 1: LC-MS/MS Chromatogram of cocainic drugs and metabolites extracted via SPE method at LOQ (1 ng/ mL)

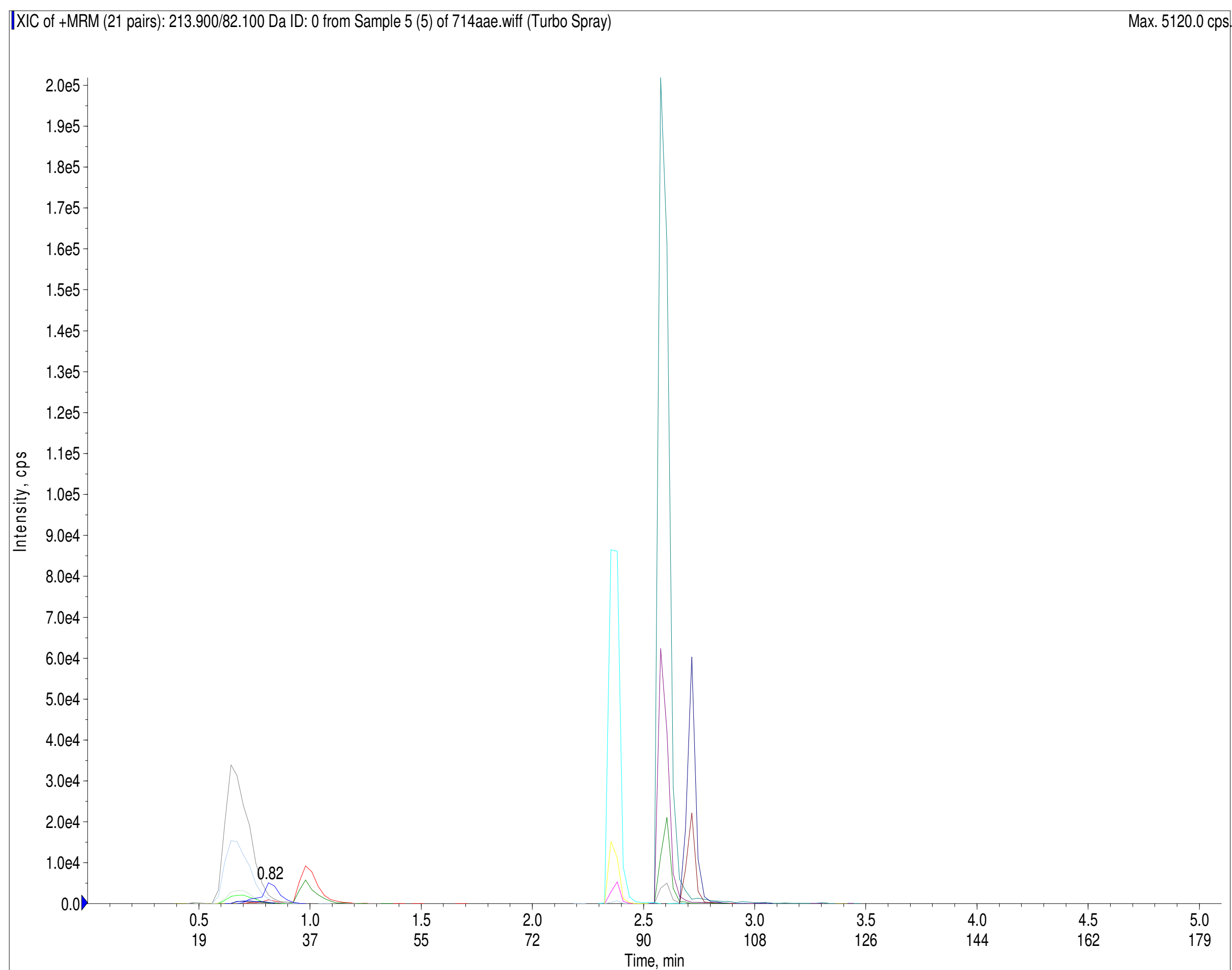

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Figure 2: LC-MS/MS Chromatogram of genuine blood extracted from a regular cocaine case.

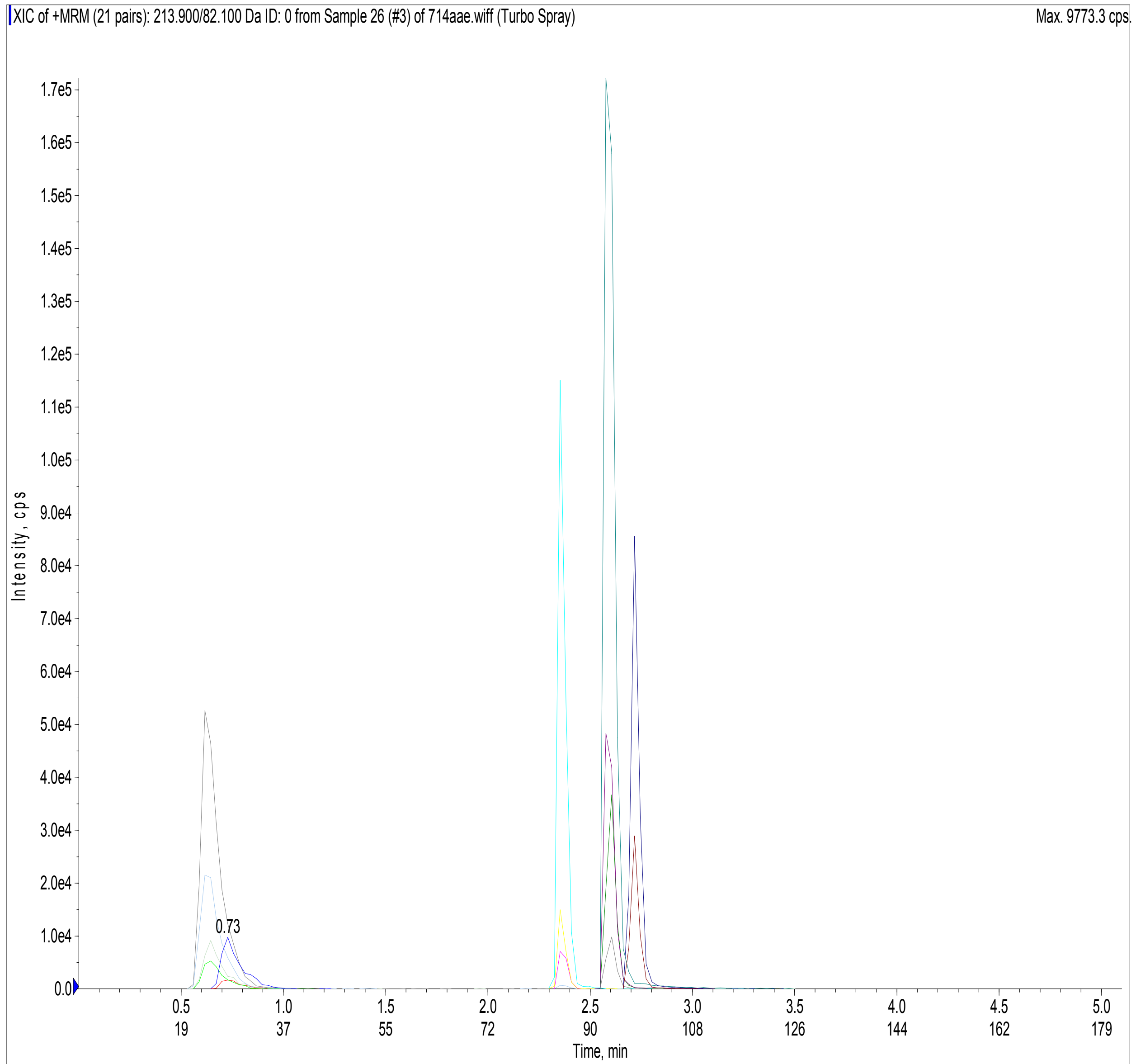

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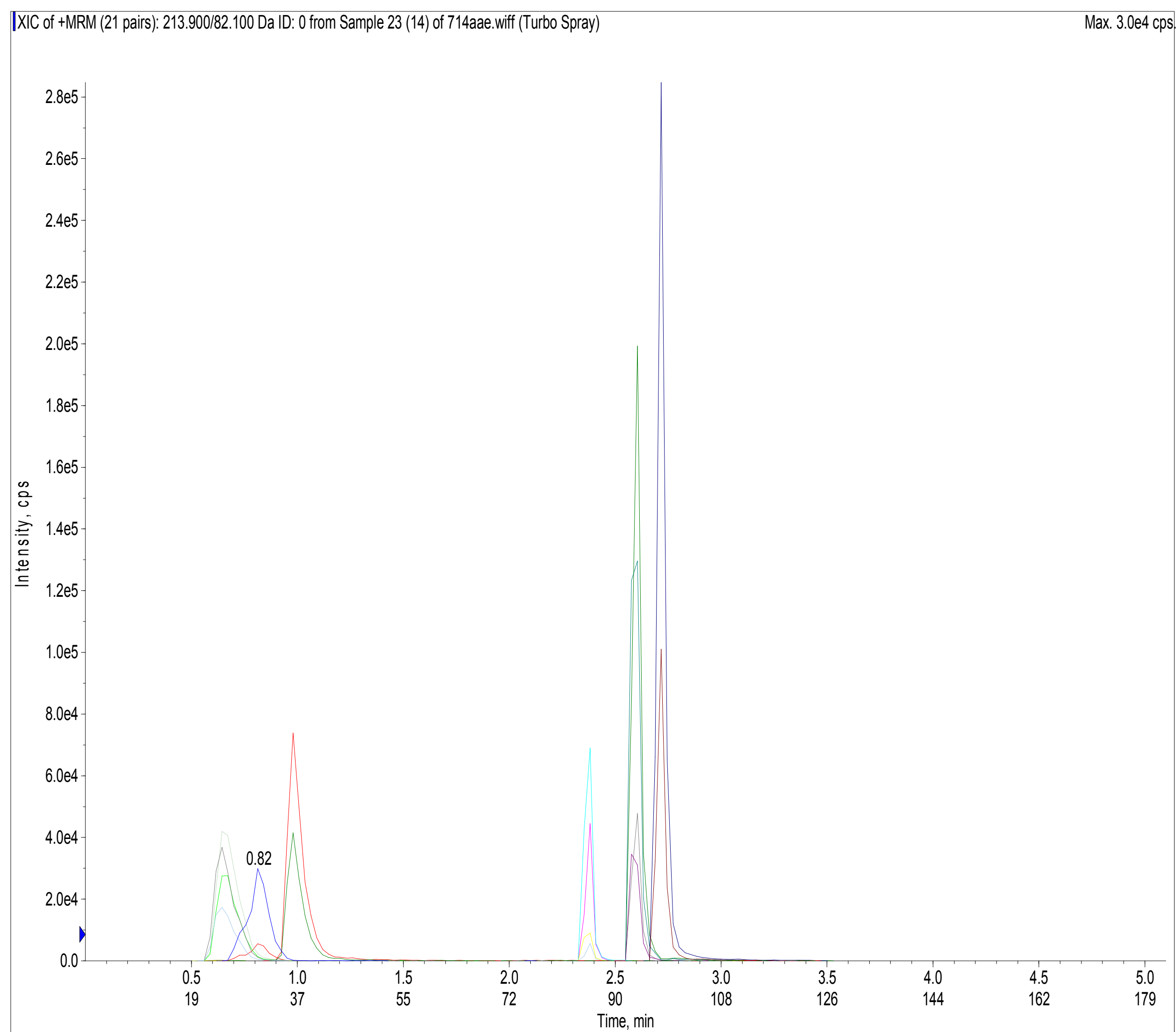
Figure 3: LC-MS/MS Chromatogram of genuine blood extracted from a "crack" cocaine case.



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