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Norcocaine and cocaethylene distribution patterns in hair samples from light, moderate, and heavy cocaine users

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Even though hair analysis often seems to be the best choice for retrospective monitoring of cocaine intake, differentiating between incorporated cocaine and external contamination is widely debated. In this study we report results obtained in 90 hair samples from addicts. All samples were analyzed for cocaine, benzoylecgonine, norcocaine, cocaethylene, and tropococaine by gas chromatography-mass spectrometry (GC-MS) techniques coupled with direct immersion solid-phase micro-extraction. Cocaine concentrations were stratified into three classes of usage: light (0.5–3 ng/mg), moderate (3.1–10 ng/mg) and heavy (10.1–40 ng/mg). The Substance Abuse and Mental Health Services Administration cut-off criteria for establishing active cocaine use were applied to the results. For all samples criteria were cocaine levels above 0.5 ng/mg (ranging from 1.63 to 39.29 ng/mg, mean 9.49 ng/mg), benzoylecgonine concentrations ≥ 0.05 ng/mg (ranging from 0.19 to 5.77 ng/mg, mean 1.40), and benzoylecgonine to cocaine % ratio ≥5% (from 6.43 to 26.09%). Norcocaine was present in 58.9% of samples (concentration range: 0.22–3.14 ng/mg) and was strongly predictive only of heavy cocaine use (sensitivity 100% for cocaine concentrations above 9.58 ng/mg). Twenty hair samples from moderate and heavy users tested positive for cocaethylene (concentration range: 0.22–1.98 ng/mg, mean 0.73 ng/mg). This study on hair samples with no chance of false positive cases highlights the very limited applications of testing minor cocaine metabolites for definitive proof of active cocaine consumption. © 2015 The Authors. *Drug Testing and Analysis* Published by John Wiley & Sons, Ltd.

Keywords: hair analysis; cocaine; norcocaine; cocaethylene; DI-SPME, GC-MS

Introduction

Cocaine (COC), a tropane alkaloid, is obtained from the leaves of the coca plant (*Erythroxylum coca*). It is a nervous system stimulant with intense addictive properties[1,2] and in recent decades has become the most popular drug worldwide. Intake of recreational powdered cocaine in Western countries is most commonly by nasal insufflation (snorting or sniffing). COC is rapidly and extensively metabolized, primarily in the liver, with only about 1% excreted unchanged in urine.[3] Since its metabolism is dominated by hydrolytic ester cleavage, excreted metabolites consist mainly of benzoylecgonine (BEG) with lesser amounts of others like ecgonine methyl ester and ecgonine. Other minor cocaine metabolites are norcocaine (NCOC), p-hydroxycocaine, m-hydroxycocaine, p-hydroxybenzoylecgonine, and m-hydroxybenzoylecgonine.[4,5] COC is often taken with ethanol producing cocaethylene (CE), a psychoactive COC homologue.[3]

During keratinization, COC and its metabolites penetrate hair where they are detected until the hair that grew during cocaine use is cut or falls out.[3] COC is the most abundant analyte in hair compared with BEG and NCOC, its polar metabolites, which are present at, respectively, 5–50% and <10% of the COC concentration.[6] Hair analysis is routinely employed in forensic science to detect and quantify drug abuse and, unlike blood and urine samples, can be applied retrospectively to determine the history and severity of an individual's drug use.[7] Cautious interpretation of test results may distinguish between passive and active COC intake. Hair samples must be washed before analysis to remove potential low-level external contamination by COC and its metabolites that could be present in the environment or in 'street' COC as a by-product of the manufacturing process. BEG might also be present, as the parent drug can form it in hair through a non-metabolic process.[8,9]

According to SAMSHA guidelines[10] COC concentrations ≥0.5 ng/mg and BEG concentrations ≥0.05 ng/mg plus a BEG to COC % ratio ≥5%, or alternatively, NCOC or CE concentrations ≥0.05 ng/mg, indicate, with high probability, active COC use. If

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the SAMSHA criteria are satisfied, there is a high probability, but no definitive proof, of COC intake.[11]

Some studies reported CE and NCOC levels when hair samples were contaminated with COC in the laboratory.[5] On the other hand, not finding these two metabolites in hair does not exclude drug usage. Biotransformation products may be missing due to lack of enzymes or co-factors such as ethanol for CE formation.[12]

In a previous study[7] we described and fully validated an analytical method that combined direct immersion (DI) solid-phase microextraction (SPME) with gas chromatography-mass spectrometry (GC-MS) to effectively detect COC, minor congeners, and levamisole. In the present study we determined concentrations of COC and its main metabolites in 90 hair samples from chronic cocaine abusers who had produced COC positive urine results and self-reported regular use. In accordance with several studies,[13,14] that compared self-reports with COC concentration in hair and our hair analysis results, we stratified three classes of usage: light (0.5–3 ng/mg), moderate (3.1–10 ng/mg), and heavy (10.1–40 ng/mg). We then evaluated distribution patterns and rates of COC, BEG, NCOC, and CE by DI-SPME-GC-MS, in these three groups of known COC-using subjects. We also report tropococaine (TPC), a contaminant of street COC, was detected in 16 of 90 hair samples.

Materials and methods

Chemicals, reagents, and standards

COC, BEG, NCOC, CE, TPC, COC-d3, BEG–d3, NCOC-d3, and CE-d3 were obtained from LGC Standards S.r.l. (Sesto San Giovanni, Italy) in 1 mg/mL or 100 μg/mL concentrations. All standards and their dilutions were stored at -20°C. Scientific grade methanol, pentafluoropropionic anhydride (PFPA), 2,2,3,3,3-pentafluoropropanol (PFPOH) and polydimethylsiloxane (PDMS) fibre (30 μm) were purchased from Sigma-Aldrich (Cambridge, UK).

A 1 mg/mL working solution containing all 5 analytes (COC, NCOC, CE, TPC, BEG) was prepared in a 2 mL glass vial. An internal standard solution containing COC-d3, NCOC-d3, BEG-d3, and CE-d3 at a concentration of 1 μg/mL was also obtained. Mixed standard solutions were used to generate calibration curves by means of a series of five injections of standards at appropriate concentrations for the expected analyte concentration range.

Hair sample collection

Laboratory procedures were in accordance with the Helsinki Declaration of 1975 as revised in 1983 and approved by Bioethics Review Board of University of Perugia (Protocol 2012-006R). All participants provided informed consent. Hair samples (N=90, male=70, female=20) were submitted for analysis of cocaine and minor congeners. Samples were obtained from volunteers who had been chronic cocaine users over the previous two years as documented by social service programmes of substance abuse monitoring. All subjects were Caucasians, all hair samples were naturally pigmented (not dyed or bleached) and colour distribution was as follows: 56 brown, 22 black, and 12 blonde.

Patients had at least four positive urine screening tests for COC (BEG cut-off 150 ng/mL) in the last three months and self-reported regular cocaine use. The most frequent COC route of administration was intra-nasal sniffing/snorting (94%), followed by smoking crack (6%). Hair samples were cut from the *posterior vertex* portion of the head as close to the scalp as possible and stored in the dark in paper envelopes at room temperature until analysis. Drug-free hair was obtained from laboratory personnel with no known illicit drug exposure.

Sample preparation

All hair samples were analyzed using a fully validated method described elsewhere.[7] Briefly, the hair samples were carefully washed and 20 μL of deuterated internal standard (mixed solution containing 1 μg/mL COC-d3; BEG-d3; NCOC-d3; CE-d3) was added. The sample was incubated with 1 mL methanol overnight at 60°C. After the methanol phase it was dried under a stream of nitrogen and then phosphate buffered saline (pH 7.4) was added before it underwent the DI-SPME sampling procedure. Liquid-liquid extraction (LLE) at pH 8.0 was performed with a mixture of chloroform/isopropanol (9/1) followed by the organic layer concentration, in accordance with in-house methods[15] only for the BEG quantification. Pentafluoropropyl derivatization of the dried extract was performed with PFPA (50 μL) and PFPOH (30 μL), at 80° C for 30 min. After evaporation of the derivatizing mixture, 25 μL of ethyl acetate was added and the final solution analyzed by GC-MS.

Instruments

The SPME device was a 30-μm polydimethylsiloxane fused silica/stainless steel fibre. Sampling was performed by DI for 1 h at 80 °C. After sampling, the fiber was thermally desorbed directly into the GC injector for 2 min.

A Focus Gas Chromatograph coupled with a DSQ (Thermo Electron Corp., Milan, Italy) operating in electron impact mode (70 eV) was used. An Equity 5 capillary column, 30 m × 0.25 mm × 0.25 mm film thickness, was processed with the following temperature program: isothermal mode for 1 min at 70 °C, then 30 °C/min to 200 °C, 5 °C/min to 250 °C, 30 °C/min to 280 °C, and then 5 min in isothermal mode. Acquisition in the selected ion monitoring mode was performed by choosing the following ions for each compound (quantification ions underlined): TPC, *m/z* 124-245-246; NCOC, *m/z* 168-289, NCOC-d3, *m/z* 171-292; COC, m/z 182-198-303, COC-d3, *m/z* 185-201-306; CE, *m/z* 196-317; CE-d3, *m/z* 199-320, BEG-PFPA derivate (300-421-316), BEG-d3 PFPA derivate (303- 424-319)

Calibration and validation parameters

Validation parameters for GC-MS analysis were specificity, linearity, lower limit of quantification (LLOQ) detection limit, accuracy, and repeatability (intra- and inter-day precision).

To evaluate specificity, five different blank hair samples collected from drug-free volunteers (with and without internal standards) were analyzed to check for peaks that might interfere with the detection of analytes. Specificity was found to be satisfactory, as the chromatograms were free of co-eluting peaks. Calibration curves were constructed from peak area ratios. Known quantities of analytes and deuterated internal standards were added to five sets of blank hair samples. Calibration curve linear ranges were 0.02–4 ng/mg for NCOC, CE, and TPC. For BEG-PFPA quantification, two curves were constructed at low (0.02–4 ng/mg) and high (4–10 ng/mg) concentrations; for COC quantification three curves were generated at low (0.02–4 ng/mg), medium (4–20 ng/mg), and high (20–40 ng/mg) concentrations. A linear response, with a coefficient of determination (r2) above 0.99, was

observed over the entire concentration range for COC, BEG, NCOC, CE, and TPC. Relative extraction recoveries were 88–94% for all analytes, as determined by comparing extracted and non-extracted spiked hair samples at three concentrations (0.2, 1, 3 ng/mg for NCOC, CE, TPC; 0.2, 2.5, 5 ng/mg for BEG; 0.2, 15, 30 ng/mg for COC).

The limit of quantification (LOQ) and limit of detection (LOD) for COC, BEG, NCOC, CE, and TPC were 0.02 and 0.01 ng/mg, respectively. Accuracy and repeatability were calculated by analyzing five independent samples of hair that was spiked with the three aforementioned concentrations of each analyte. Accuracy was expressed as the observed mean value percent deviation from the nominal value and repeatability as the relative standard deviation; results were satisfactory for both. Intra- and inter-day coefficients of variation were determined by analyzing five replicates at the three aforementioned concentrations for each analyte either during the same run (intra-day) and on five different days (inter-day). They ranged below 15% and 20.5%, respectively.

Statistical analysis

Pearson's chi-squared test ($\chi 2$) evaluated likelihood of chance difference between sets for all variables reported in the tables.

To assess NCOC sensitivity and specificity as a marker of active COC use, sensitivity and specificity were plotted against probability cut-off *c*, after logistic regression estimation (Figure 1). Let *pj* be the predicted probability of a positive outcome and *yj* be the actual outcome, which we will treat as 0 or 1, although Stata treats it as 0 and non-0, excluding missing observations. A prediction is classified as positive if *pj c*; otherwise it is classified as negative. The classification is correct if it is positive and $yj = 1$ or if it is negative and $yj = 0$. Sensitivity is the fraction of $yj = 1$ correctly classified observations. Specificity is the percentage of $yj = 0$ correctly classified observations.

Values are then sorted on sensitivity, on specificity and probability, and the closest sensitivity and specificity values are listed. Using 0.05 (the associate probability values at the closest line) for the optimal cut-off point, a classification matrix is created.[16]

Finally, sensitivity and specificity are used for constructing an ROC curve defined as the sensitivity by inverse specificity. A model with no predictive value will have a slope of 1, resulting in an ROC of 0.5.

All estimates were performed using the statistical program STATA 13.1 (Stata Corp Ltd, College Station, Texas, USA).

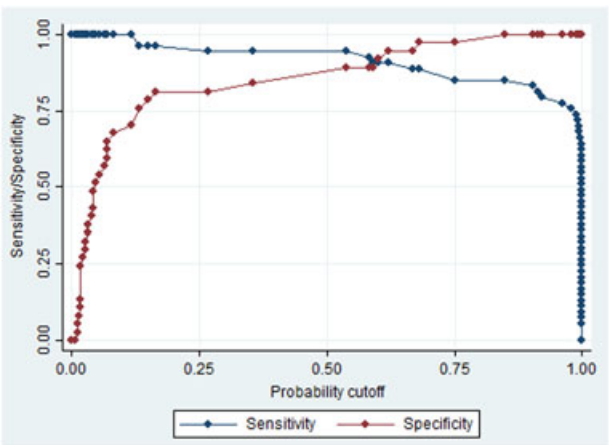

Figure 1. Sensitivity and specificity versus probability cut-off.

Results and discussion

All 90 hair samples were collected from cocaine addicts who had produced COC-positive urine tests and self-reported regular cocaine use mainly by nasal insufflation (94%). Therefore COC positive hair results were assumed to be free of external contamination and all samples were evaluated in the study of BEG, NCOC, and CE distribution patterns. Hair colour appears to affect accumulation and retention of drugs such as COC, since black or brown hair with its high eumelanin levels accumulates more basic type drugs, such as COC, than blond, gray, or red hair.[17] Dark hair also appears to be more susceptible to drug incorporation from *in vitro* contamination than light-coloured hair.[5] All the hair samples we analyzed belonged to Caucasian subjects, were naturally pigmented, and prevalently brown or black in colour (86.7%), so the influence of hair colour on the present drug test outcome can be reasonably considered the same.

Methanol was used as the hair extraction solvent because it efficiently detaches analytes from the hair shaft with no need for the extreme pH conditions that would invariably hydrolyze COC to BEG, thus producing *in vitro* metabolites.[8] The analytical procedure (DI-SPME-GC-MS), which had little sample manipulation and the required specificity, accuracy, and sensitivity at picogram concentrations to detect minor COC metabolites in the keratin matrix, can be performed routinely in forensic toxicological analysis. In accordance with Society of Hair Testing (SoHT) recommended criteria[18] we washed hair samples, identified COC metabolites (BEG, NCOC, CE), and applied metabolite-to-parent drug ratios (BEG to COC $\geq$ 5%) and threshold values (COC$\geq$ 0.5 ng/mg, BEG, NCOC, CE$\geq$ 0.05 ng/mg).

Figure 2 shows ion chromatograms for TPC, NCOC, CE, BEG-PFPA, and COC from a real hair sample.

Table 1 reports the results of hair analysis from 90 real COC users (COC, BEG, NCOC, CE, TPC amounts and % BEG/COC, %NCOC/COC, %NCOC/BEG, %CE/COC, %CE/BEG ratio). COC levels were above 0.5 ng/mg (ranging from 1.63 to 39.29 ng/mg) in all samples. Mean COC concentration was 9.49 ng/mg, indicating that most patients used COC regularly.[13,19] BEG levels, which were determined in all hair samples, were above 0.05 ng/mg (ranging from 0.19 to 5.77 ng/mg, mean value 1.40). As the COC/BEG percentage ranged from 6.43 to 26.09% (mean value 13.18%), it was always higher than 5% recommended by the SOHT as a positive COC result. In 82.2% of hair samples BEG levels were above 10% of COC levels. Any metabolites detected at this level probably derived from an endogenous source and from COC ingestion.[8]

Our data concur with hair testing studies that reported lower levels of BEG than COC.[13,17,19] The basic drug COC has a greater affinity for melanine than for BEG, its amphoteric metabolite.[19] Besides, cocaine's greater lipophilicity probably allows more COC than BEG to be incorporated.[19] Other target analytes in hair that could demonstrate *in vivo* COC use include NCOC and CE with cut-off levels $\geq$ 0.05 ng/mg.

COC is N-demethylated by cytochrome P450 3A4 to the pharmacologically active NCOC.[20] Although the extent of N-demethylation of COC appears to vary widely among species[21] in humans it was found to be 2–6%.[22] NCOC was detected in 53/90 hair samples (58.9%), with levels ranging from 0.22 to 3.14 ng/mg (mean 1.21 ng/mg). The mean NCOC to COC ratio was 8.05 % (range: 3.98–12.94). Although NCOC was reported to be present in hair at $<$10 % of COC concentration,[6] the % NCOC/COC was over 10 % and up to 13% in 6 cases. When expressed as a percentage of BEG, NCOC ranged from 25.29 to 75.27% (mean: 52.73%).

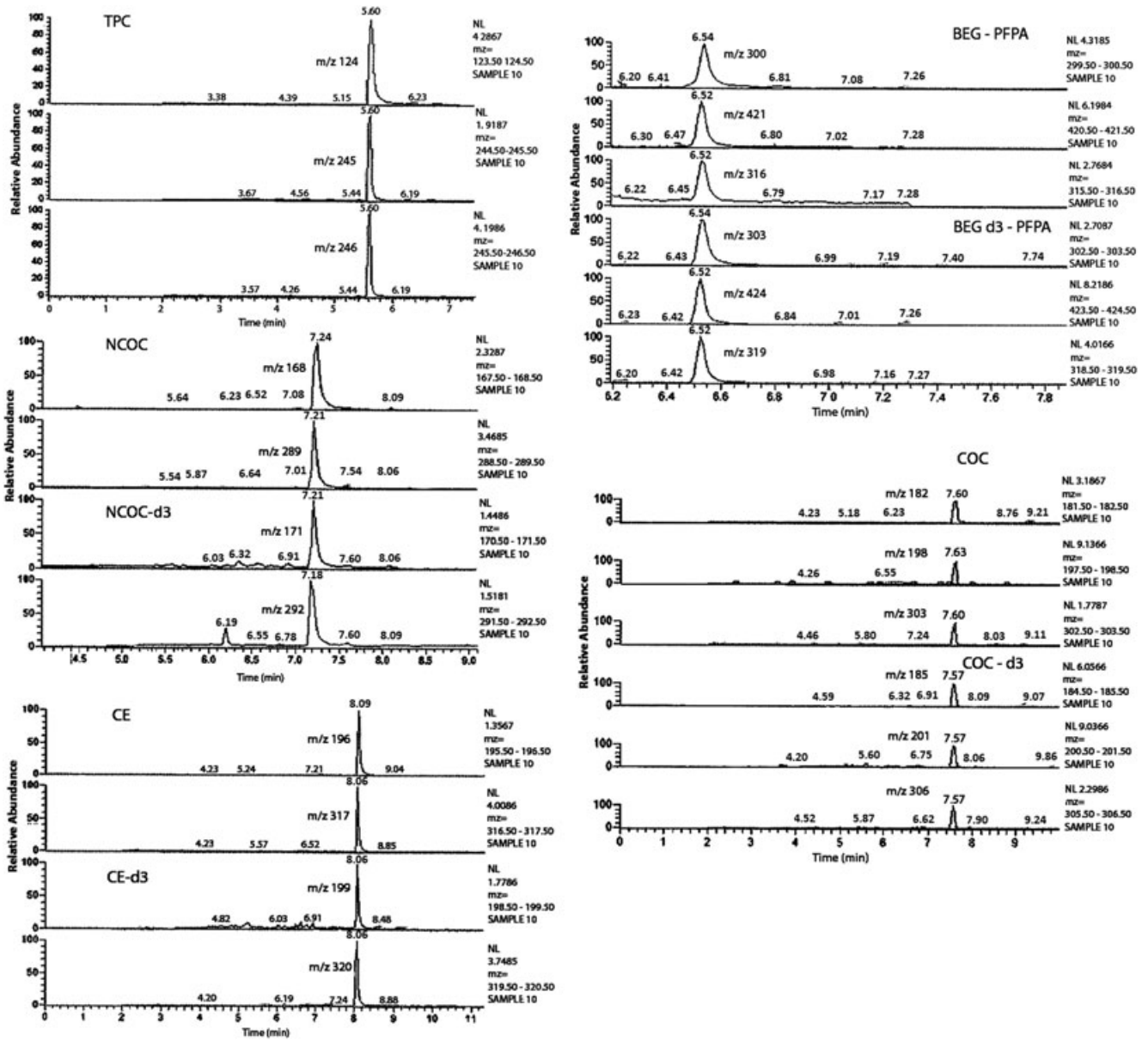

Figure 2. Ion chromatograms of a hair sample positive for TPC, NCOC, CE, BEG-PFPA, and COC.

CE was found in 20 hair samples (22.2%), with levels ranging from 0.22 to 1.98 ng/mg (mean 0.73 ng/mg). Since CE is considered a metabolic marker rather than a contaminant its presence in hair samples can be accepted as sufficient proof of COC intake. In fact, a CE/COC ratio over 0.02 was recently proposed as an additional new criterion for a positive hair test.[19] However, since COC is transesterified by hepatic carboxylesterase into CE only when COC and alcohol have been ingested simultaneously,[19] CE cannot be considered a universal marker of active COC use.

The CE/COC ratio was ≥ 0.02 in all our samples but as few samples were CE positive, this parameter cannot be considered as significantly linked to COC intake. Like many other illicit drugs, street samples of COC contain impurities, contaminants, diluents, and adulterants[7] and COC extraction from coca leaves can result in the presence of impurities such as natural alkaloids like TPC, hydroxycocaine, cis-cinnamoylcocaine, trans-cinnamoylcocaine, etc.[23] Interestingly we reported we found TPC in 16 hair samples (17.8%) at a mean concentration of 0.41 ng/mg (range: 0.20–1.10 ng/mg), even though it cannot be considered a marker of active COC use since it is not a product of *in vivo* COC metabolism. The few positive CE results in our hair samples did not appear to correlate with the amount of COC and the presence of minor metabolites.

Based on several studies that attributed COC levels in hair to different ranges of COC use, we stratified our results into three classes of COC usage: light (0.5–3 ng/mg), moderate (3.1–10 ng/mg) and heavy (10.1–40 ng/mg) so as to have the means for an in-depth analysis of COC metabolite distribution patterns and metabolite to COC ratios. Twenty-one patients (23.3%) fell within the COC light consumption range with the minimum COC level in hair being 1.63 ng/mg. In this group NCOC and CE were never detected but BEG was always found and the % BEG/COC ratio was always above 6.43. According to Poon *et al.*[6] our results indicate that NCOC was always absent in hair samples with COC concentrations below 3 ng/mg, showing it is not a useful marker of active COC use in the population of occasional users. In these cases, the very low or

Table 1. Concentrations of cocaine and metabolites in hair from 90 subjects

SAMPLE	COC (ng/mg)	BEG (ng/mg)	NCOC (ng/mg)	CE (ng/mg)	TPC (ng/mg)	% BEG/COC RATIO	%NCOC/COC RATIO	%NCOC/BEG RATIO	%CE/COC RATIO	%CE/BEG RATIO
HAIR-1	2.80	0.30	ND	ND	ND	10.71	—	—	—	—
HAIR-2	18.27	3.31	1.43	ND	0.50	18.12	7.83	43.20	—	—
HAIR-3	2.32	0.21	ND	ND	ND	9.05	—	—	—	—
HAIR-4	9.75	1.25	0.40	ND	ND	12.82	4.10	32.00	—	—
HAIR-5	12.14	1.98	1.12	ND	ND	16.31	9.23	56.57	—	—
HAIR-6	8.56	1.21	0.41	ND	ND	14.14	4.79	33.88	—	—
HAIR-7	3.86	0.47	0.29	ND	0.30	12.18	7.51	61.70	—	—
HAIR-8	2.63	0.21	ND	ND	ND	7.98	—	—	—	—
HAIR-9	5.78	0.59	ND	ND	ND	10.21	—	—	—	—
HAR-10	8.24	1.28	0.76	0.42	0.33	15.53	9.22	59.38	5.10	32.81
HAIR-11	9.87	1.22	0.66	0.31	ND	12.36	6.69	54.10	3.14	25.41
HAIR-12	4.58	0.59	ND	ND	ND	12.88	—	—	—	—
HAIR-13	22.63	3.44	2.28	ND	0.39	15.20	10.08	66.28	—	—
HAIR-14	17.87	2.78	1.41	ND	ND	15.56	7.89	50.72	—	—
HAIR-15	3.59	0.54	0.29	0.24	ND	15.04	8.08	53.70	6.69	44.44
HAIR-16	18.21	3.27	1.65	ND	0.78	17.96	9.06	50.46	—	—
HAIR-17	6.73	0.99	0.48	ND	0.18	14.71	7.13	48.48	—	—
HAIR-18	13.14	2.29	1.70	0.44	ND	17.43	12.94	74.24	3.35	19.21
HAIR-19	7.73	0.92	0.41	ND	0.48	11.90	5.30	44.57	—	—
HAIR-20	3.31	0.36	ND	ND	ND	10.88	—	—	—	—
HAIR-21	6.57	0.88	0.40	ND	ND	13.39	6.09	45.45	—	—
HAIR-22	2.10	0.21	ND	ND	ND	10.00	—	—	—	—
HAIR-23	5.53	0.71	0.32	ND	ND	12.84	5.79	45.07	—	—
HAIR-24	15.66	2.40	1.22	ND	ND	15.33	7.79	50.83	—	—
HAIR-25	5.11	0.72	0.32	ND	ND	14.09	6.26	44.44	—	—
HAIR-26	20.79	2.96	1.86	ND	ND	14.24	8.95	62.84	—	—
HAIR-27	10.80	1.93	1.28	0.38	ND	17.87	11.85	66.32	3.52	19.69
HAIR-28	5.53	0.87	0.22	ND	ND	15.73	3.98	25.29	—	—
HAIR-29	8.44	1.20	0.56	ND	ND	14.22	6.64	46.67	—	—
HAIR-30	9.57	1.23	0.62	ND	ND	12.85	6.48	50.41	—	—
HAIR-31	2.20	0.21	ND	ND	ND	9.55	—	—	—	—
HAIR-32	27.29	3.81	2.57	ND	ND	13.96	9.42	67.45	—	—
HAIR-33	16.98	2.83	1.65	0.48	ND	16.67	9.72	58.30	—	—
HAIR-34	1.99	0.21	ND	ND	ND	10.55	—	—	—	—
HAIR-35	3.59	0.44	ND	ND	0.23	12.26	—	—	—	—
HAIR-36	4.58	0.56	ND	ND	ND	12.23	—	—	—	—
HAIR-37	21.93	3.58	2.25	0.79	0.56	16.32	10.26	62.85	3.60	22.07
HAIR-38	6.21	1.62	0.57	ND	ND	26.09	9.18	35.19	—	—
HAIR-39	3.78	0.52	ND	ND	ND	13.76	—	—	—	—
HAIR-40	5.24	0.71	0.28	ND	ND	13.55	5.34	39.44	—	—
HAIR-41	2.11	0.21	ND	ND	ND	9.95	—	—	—	—
HAIR-42	3.17	0.36	ND	ND	ND	11.36	—	—	—	—
HAIR-43	2.79	0.21	ND	ND	ND	7.53	—	—	—	—
HAIR-44	5.26	0.68	ND	ND	ND	12.93	—	—	—	—
HAIR-45	6.66	0.82	0.27	ND	0.48	12.31	—	—	—	—
HAIR-46	2.99	0.20	ND	ND	ND	6.69	—	—	—	—
HAIR-47	2.87	0.21	ND	ND	ND	7.32	—	—	—	—
HAIR-48	2.51	0.22	ND	ND	ND	8.76	—	—	—	—
HAIR-49	2.11	0.19	ND	ND	ND	9.00	—	—	—	—
HAIR-50	4.29	0.46	ND	ND	ND	10.72	—	—	—	—
HAIR-51	2.62	0.20	ND	ND	ND	7.63	—	—	—	—
HAIR-52	3.11	0.20	ND	ND	ND	6.43	—	—	—	—
HAIR-53	10.87	1.99	0.72	ND	0.20	18.31	6.62	36.18	—	—
HAIR-54	3.59	0.61	0.26	ND	ND	16.99	—	—	—	—
HAIR-55	2.78	0.23	ND	ND	ND	8.27	—	—	—	—
HAIR-56	1.87	0.20	ND	ND	ND	10.70	—	—	—	—

(Continues)

Table 1. (Continued)

SAMPLE	COC (ng/mg)	BEG (ng/mg)	NCOC (ng/mg)	CE (ng/mg)	TPC (ng/mg)	% BEG/COC RATIO	%NCOC/COC RATIO	%NCOC/BEG RATIO	%CE/COC RATIO	%CE/BEG RATIO
HAIR-57	7.24	0.88	0.44	ND	ND	12.15	6.08	50.00	—	—
HAIR-58	12.30	2.62	1.20	ND	0.28	21.30	9.76	45.80	—	—
HAIR-59	5.49	0.64	ND	ND	ND	11.66	—	—	—	—
HAIR-60	18.46	2.75	1.43	ND	ND	14.90	7.75	52.00	—	—
HAIR-61	22.53	3.49	2.22	1.80	ND	15.49	9.85	63.61	7.99	51.58
HAIR-62	3.59	0.47	ND	ND	ND	13.09	—	—	—	—
HAIR-63	17.36	2.81	1.64	0.69	ND	16.19	9.45	58.36	—	—
HAIR-64	5.35	0.91	0.37	0.25	ND	17.01	6.92	40.66	4.67	27.47
HAIR-65	2.11	0.20	ND	ND	ND	9.48	—	—	—	—
HAIR-66	22.56	3.39	2.55	0.94	ND	15.03	11.30	75.22	4.17	27.73
HAIR-67	16.51	2.46	1.22	ND	ND	14.90	7.39	49.59	—	—
HAIR-68	2.11	0.21	ND	ND	ND	9.95	—	—	—	—
HAIR-69	2.79	0.22	ND	ND	ND	7.89	—	—	—	—
HAIR-70	10.29	1.73	0.92	0.27	ND	16.81	8.94	53.18	2.62	15.61
HAIR-71	24.89	3.85	2.35	1.28	0.47	15.47	9.44	61.04	5.14	33.25
HAIR-72	3.68	0.41	ND	ND	ND	11.14	—	—	—	—
HAIR-73	17.91	2.99	1.58	ND	ND	16.69	8.82	52.84	—	—
HAIR-74	8.88	0.99	0.36	0.22	ND	11.15	4.05	36.36	2.48	22.22
HAIR-75	1.93	0.21	ND	ND	ND	10.88	—	—	—	—
HAIR-76	5.29	0.60	ND	ND	ND	11.34	—	—	—	—
HAIR-77	34.76	5.62	3.11	1.69	0.66	16.17	8.95	55.34	4.86	30.07
HAIR-78	25.11	3.65	2.32	1.24	1.10	14.54	9.24	63.56	4.94	—
HAIR-79	1.63	0.20	ND	ND	ND	12.27	—	—	—	—
HAIR-80	39.29	5.77	3.14	1.98	0.35	14.69	7.99	54.42	5.04	34.32
HAIR-81	10.61	1.62	0.86	0.53	ND	15.27	8.11	53.09	5.00	—
HAIR-82	16.28	2.71	1.10	ND	ND	16.65	6.76	40.59	—	—
HAIR-83	15.46	2.37	1.53	ND	ND	15.33	9.90	64.56	—	—
HAIR-84	3.19	0.37	ND	ND	ND	11.60	—	—	—	—
HAIR-85	11.78	1.82	1.37	ND	ND	15.45	11.63	75.27	—	—
HAIR-86	8.67	0.92	0.49	0.26	ND	10.61	5.65	53.26	3.00	28.26
HAIR-87	2.47	0.21	ND	ND	ND	8.50	—	—	—	—
HAIR-88	3.18	0.35	ND	ND	ND	11.01	—	—	—	—
HAIR-89	32.54	4.69	2.92	ND	ND	14.41	8.97	62.26	—	—
HAIR-90	12.37	1.92	1.19	0.38	ND	15.52	9.62	61.98	3.07	19.79
MEAN	9.49	1.40	1.21	0.73	0.41	13.18	8.05	52.73	4.35	28.37

low COC levels in hair could lead to difficulties in attributing the positive result to drug intake rather than external contamination, particularly in high-risk populations such as police officers, drug-dependent males or their family members and individuals who are involved in illicit COC manufacturing or distribution.[11,12]

Thirty-eight patients (42.22%) belonged to the category of moderate COC consumers. In this group, NCOC was present in 22 hair samples (57.89%).

The classification matrix shows that the model correctly classifies 81.11 % of the cases. This good value provides evidence that the model is well fitted, which was confirmed by the 0.89 area under the ROC curve (also known as Harrel's C statistic).

The overall positive predictive value of NCOC positivity was 58.5%, showing NCOC was not a highly sensitive marker of systemic COC intake.

Thirty-one patients (34.44%) had COC concentrations above 10 ng/mg, NCOC was found in all samples. For COC concentrations above 9.58 ng/mg, sensitivity was 100%, specificity was 62.7% and the positive predicted value was 75.71%. So COC concentrations above 9.58 ng/mg are strongly predictive of NCOC positivity. Interestingly this level was very close to the COC mean concentration (9.49 ng/mg) that we found in the 90 hair samples we analyzed. Our data concur Poon *et al.*'s findings,[6] indicating NCOC is a highly sensitive marker only for heavy COC use. Table 2 summarizes the frequency of NCOC detection in the three ranges of COC consumption.

Table 2. Frequency of NCOC determination in three classes of COC consumption ($p<0.00001$).

COC categories				
NCOC	0.5–3 ng/mg	3.1–10 ng/mg	>10 ng/mg	TOTAL
NEGATIVE	21	16	0	37
	100%	42.11%	0%	41.11%
POSITIVE	0	22	31	53
	0%	57.89%	100%	58.89%
TOTAL	21	38	31	90
	100%	100%	100%	100%

Pearson chi2(2)=51.7381 Pr =0.000.

Table 3. Frequency of CE determination in NCOC positive hair samples ($p<0.00001$).

CE			
NCOC	NEGATIVE	POSITIVE	TOTAL
NEGATIVE	37	0	37
	52.86%	0%	41.11%
POSITIVE	33	20	53
	47.14%	100%	58.89%
TOTAL	70	20	90
	100%	100%	100%

Pearson chi2(2)=17.9515 Pr =0.000.

Interestingly CE was found together with NCOC in the 20 CE positive hair samples (6 cases in medium range COC usage, 14 in the high). Table 3 correlates the frequency of CE detection with NCOC positivity. Unfortunately, the number of CE positive samples was too low for further analysis but it seems clear that, as with NCOC, the probability of CE positivity increases significantly when cocaine use is heavy.

In conclusion, COC hair analysis in 90 proven COC users with no possibility of false positive cases, showed that in all three classes of cocaine users (light, moderate and heavy) the following criteria are always fulfilled: COC concentration ≥0,5 ng/mg, BEG concentration ≥0.05 ng/mg. BEG to COC % ratio > 6.43 was observed in all. The presence of minor metabolites as proof of COC usage has very limited applications. NCOC emerged as a highly sensitive marker only for COC concentrations above 9.58 ng/mg (95% confidence interval from 6.64 to 12.26 ng/mg) which corresponds to frequent drug use. CE was found in 20 hair samples, but since it is present only after combining COC and alcohol consumption, it cannot be considered a universal marker of active COC usage.

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