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A Novel Method for the Detection of Cocaine in Hair using a Freeze/ Thaw Method and GC/ MS Analysis

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Abstract: In order to create an efficient and less strenuous method for the detection of cocaine in hair, a simple freeze-thaw extraction method was developed and coupled to gas chromatography/mass spectrometry (GC/MS). GC/MS analysis parameters were determined by testing solutions of cocaine and two of its metabolites, cocaethylene and ecgonine methyl ester. Ions from these compounds and two pyrolysis products, anhydroecgonine methyl ester and 3-ethoxy-4-methoxyphenethylamine, were chosen in order to test samples using selected ion monitoring (SIM). The qualitative limit of detection for anhydroecgonine methyl ester, 3-ethoxy-4-methoxyphenethylamine, ecgonine methyl ester, cocaine, and cocaethylene were 0.1, 0.1, 0.50, 0.05, and 0.05 ng, respectively.

Using cocaine-positive hair samples from Willow Laboratories (Lynn, MA, USA), an extraction method was developed where tested hairs were washed, segmented, covered in acetonitrile, frozen and thawed for five cycles, and evaporated until a minimal amount of liquid remained. Results were obtained with 2ul of extract using GC/MS with SIM. Ions specific for cocaine were identified in the cocaine-positive hair samples, allowing for cocaine-positive hairs to be distinguished from cocaine-negative hair samples. Utilizing this method, results can be obtained with as little as 10mg of hair, though 20mg is recommended. A blind testing study with samples from Willow Laboratories provided results that were consistent with those obtained by other methodologies employed by Willow Laboratories.

Thus, the freeze/thaw method via GC/MS analysis is a viable method that significantly decreases sample preparation by improving upon the ease of extraction for the detection of cocaine in hair.

Key Words: Hair; Cocaine; Extraction; Freeze/thaw; Gas chromatography/mass spectrometry.

There is a long list of analytical procedures used for analyzing hair for the presence of drugs; however, many of these procedures involve lengthy and strenuous sample preparation. This study investigated the use of a novel extraction technique followed by GC/MS analysis to detect cocaine in hair aimed to improve upon the complexity of previous sample preparation methods.

In order to understand how the detection of drugs in hair is possible, the concept of drug incorporation into hair must be understood. The current model of incorporation explains that not only do drugs enter the hair follicles from capillaries near the hair bulb via passive diffusion, but they also can be absorbed from secretions of the various glands that are present near the hair shaft itself.

Therefore, drugs can be absorbed from the environment if they are deposited onto the hair which could result from contamination [1-3]. This possibility of contamination is typically remedied during sample preparation which is composed of three main steps: collection of the specimen, sample washing, and extraction or digestion. The third and last step extracts and purifies the drug from the hair matrix for analysis. Extraction is the step of interest in this study.

Most methods for the analysis of drugs in hair involve complex sample preparation with time frames ranging from a few hours to a few days before analysis can be performed [4]. The most common methods of extraction for drugs in hair include liquid-liquid extraction

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and solid-phase extraction which can be tedious and time consuming as there are numerous variables to consider [5].

It should be noted that the metabolites of cocaine are more polar than the parent drug (cocaine).Thus, unlike other biological matrices such as blood, the parent drug will be present in higher concentrations than the metabolites because the less polar cocaine will pass through membranes more easily than its metabolites [6].

Historically, radioimmunoassay (RIA) was the most common screening technique used for the detection of cocaine in hair; however, recently enzyme-linked immunosorbant assay (ELISA) has replaced RIA as the method of choice mainly because it does not involve the use of radioactively labeled materials [7].

Fluorescence polarization immunoassay (FPIA) and microplate enzyme immunoassay (EIA) have also been documented as screening tests for cocaine in hair, though again, immunoassays can take many hours of sample preparation and even longer to complete the analysis [4, 8-11]. Immunoassays must also adhere to certain criteria: cross-reactivity with the parent drug and metabolites, no matrix interference from the hair, and application of proper cut-off values for drug concentrations in hair [4].

High-performance liquid chromatography (HPLC) has been offered as an alternative to immunoassays. Though less time consuming than immunoassays, method development for HPLC can be tedious and sensitivity can be problematic [6].

This study searches for an extraction method which improves the ease of sample preparation. Current extraction techniques include digestion with alkali, acid extraction, and enzymatic treatment, all of which involve incubation for a lengthy period of time [6]. Many methods of sample preparation involve various reagents and incubation times as long as 18 hours [11, 12]. This study simplifies the extraction technique by employing a single solvent in a freeze/thaw extraction followed by GC/MS analysis and improves upon current methods in the literature by sample preparation strain and time.

MATERIALS AND METHODS

Instrumentation

Separation and detection of analytes were carried out using an Agilent 6890N Gas Chromatograph with a 5973N Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA).

Gas chromatography was carried out using a ZB-5HT InfernoTM 30m column (95% dimethylpolysiloxane/5% phenyl, Phenomenex, Torrance, CA, USA) with 5 Guardian (5m guard column placed before the column).

Instrumentation Parameters and Drug Standards

Analysis of a standard drug mix was performed using the following gas chromatography parameters specified in Table 1.

Table 1. Summary of gas chromatography parameters

Oven Parameters			Front Inlet Parameters
Initial Temperature: 40°C			Mode: Splitless
Initial Time: 2.00min			Initial Temperature: 250°C
Ramps:			Pressure: 18.54 psi
Rate	Final Temp	Final Time	Gas Type: Helium
10.00°C/min	295°C	22.50min	
Post Temperature: 40°C			
Post Time: 0.00min			
Run Time: 50.00min			

To prepare the drug standard mixture, 100µl of each drug standard solution (1mg/ml of ecgonine methyl ester, cocaine, and cocaethylene respectively, prepared in acetonitrile) were combined, with the resulting solution brought to 1ml with acetonitrile to give a 100ng/ul concentration standard mix. This drug mix was analyzed using GC/MS (1µL) to determine the retention times of each drug in the mixture. The gas chromatography parameters in Table 1 were used for all analyses and this method will be referred to henceforth as the splitless method.

Scanning parameters for the mass spectrometer were set from 40-450amu. Three to four ions descriptive for cocaine (COC), cocaethylene (CE), ecgonine methyl ester (EME), and two pyrolysis products, anhydroecgonine methyl ester (AEME) and 3-ethoxy-4-methoxyphenethylamine, were chosen and a selected ion monitoring (SIM) method created in order to improve the sensitivity of the method.

Quality Control Parameters

Before each sample run, a column blank or solvent blank was performed to ensure no carry-over of previous samples. For quality control, the drug standard mix was analyzed prior to each use of the instrument.

Qualitative Limit of Detection

The qualitative limit of detection was determined using GC/MS and the splitless SIM method using the drug standard mix. The following concentrations of the drug standard mix were prepared for analysis: 100ng/µl, 25ng/µl, 10ng/µl, 1ng/µl, 0.1ng/µl, 0.5ng/µl, 0.25ng/µl, 0.1ng/µl, 0.05ng/µl, and 0.025ng/µl. All were run in triplicate and injected in 2µl amounts. The qualitative limit of detection determined was based upon the least sensitive ion.

Hair Samples

The use of hairs taken from human subjects was approved by the Cedar Crest College Institutional Review Board under number 1037-08. The identities of the human subjects were unknown to the authors.

Extraction Procedure: Freeze/ Thaw Method

Entire hairs were used from both positive and negative samples and cut in 2-5mm sections. Positive samples were tested at the following quantities: 30mg, 20mg, and 10mg. All negative samples were tested at a quantity of 30mg. The hair was washed in the following: dichloromethane (1-2 minutes), deionized water (brief washing), acetonitrile (brief washing). In each washing, enough of each solvent was added to cover the hair and was removed once complete. Enough acetonitrile was then added to cover the hair completely. The samples were covered and placed into a -80°C freezer. Once frozen (about 10 minutes), the samples were removed and thawed. After thawing was complete (about 1 minute), the samples were placed into the freezer and the process was repeated for a total of five freeze/thaw cycles. To provide a further improved sample preparation technique, this method could also be performed using liquid nitrogen to speed the process, resulting in a more efficient extraction. Once the cycles were complete, the samples were placed into a 100°C oven where they were evaporated to the point where only a very small amount of acetonitrile remained. Two (2) microliters of this liquid was then analyzed using GC/MS and the splitless SIM method.

Blind Testing

Twelve hair samples were obtained from Willow Laboratory and tested to determine if any contained cocaine metabolites. About 20-25mg of each hair was extracted using the freeze/thaw method. Each was analyzed using the GC/MS and splitless SIM method, after which the results were compared to those found by Willow Laboratory via a different method.

RESULTS

Drug Standard Mix Data

Figure 1 shows a total ion chromatogram (TIC) of 1µL of the 100ng/µl drug standard mix analyzed with the GC/MS in which five compounds are labeled peaks 1-5. AEME and 3-ethoxy-4-methoxyphenethylamine are pyrolysis products observed from the analysis of EME, COC, and CE. Though the retention times of these compounds may seem lengthy, the GC parameters can be modified to decrease retention time and provide a more efficient method. As this was a preliminary study, the main goal was to provide reduced complexity of sample preparation for the extraction method.

Ion Selection

Four ions were chosen for COC and CE whereas three were chosen for AEME, 3-ethoxy-4-methoxyphenethylamine, and EME. A selected ion monitoring (SIM) method was created based upon the ions chosen. Table 2 summarizes the ions chosen as well as the SIM method screening time.

Figure 2 shows representative extracted ion profiles for each compound from the drug standard mix using the ions of interest for each compound using the splitless SIM method.

Qualitative Limit of Detection

The qualitative limit of detection (LOD) was determined for all five compounds. The qualitative detection limits for each compound are based upon the least sensitive ion, thus the disappearance of one of the chosen ions indicated the qualitative limit of detection

Figure 1. TIC of the drug standard mix utilized (1µL). Peaks: AEME (1), 3-ethoxy-4-methoxyphenetthylamine (2), EME (3), COC (4), CE (5).

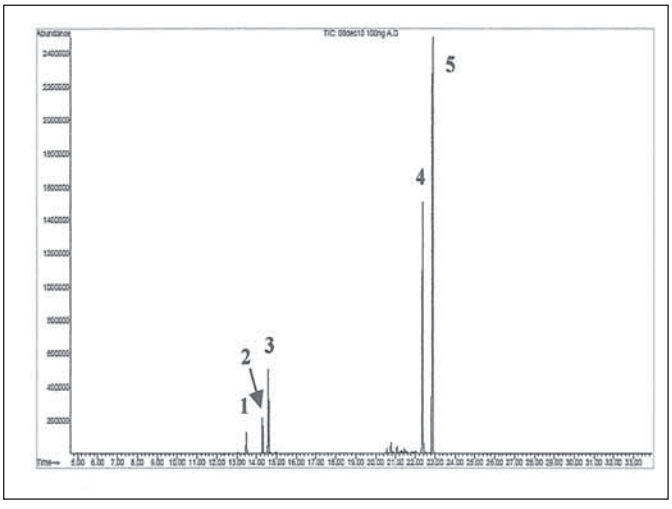

Table 2. The ions chosen for each compound along with the SIM method screening time used for each (the molecular ion for cocaine and cocaethylene are shown in bold).

Product	Ions Chosen	SIM Method
Anhydroecgonine methyl ester (AEME)	152, 166, 181	4.50 min (after solvent delay)
3-ethoxy-4-methoxyphenethylamine	138, 166, 195	14.00 min
Ecgonine methyl ester (EME)	96, 182, 199	14.50 min
Cocaine (COC)	181, 182, 272, **303**	18.00 min
Cocaethylene (CE)		22.60 min

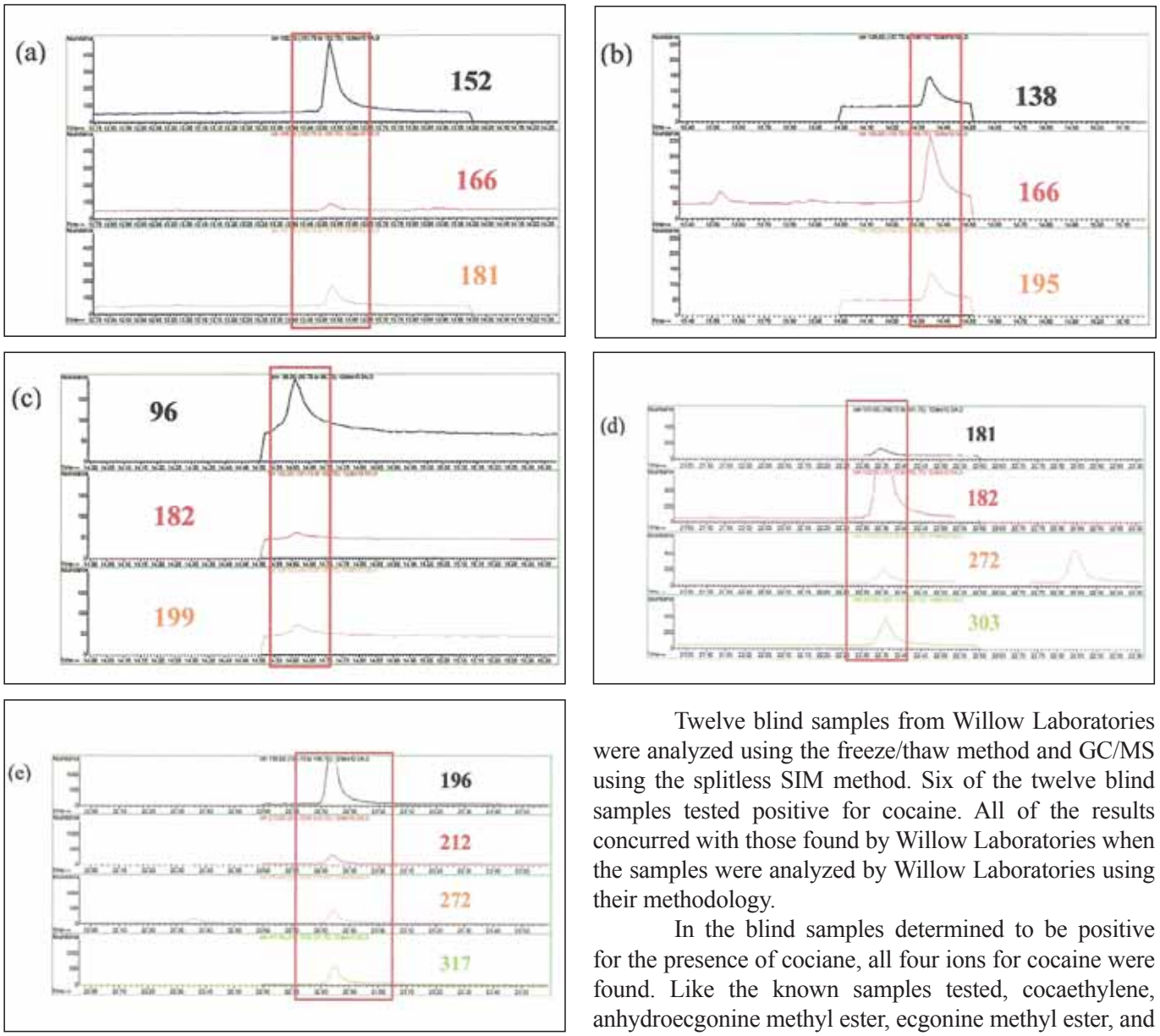

Figure 2. EIP from the drug standard mix for the chosen ions for anhydroecgonine methyl ester (a), 3-ethoxy-4-methoxyphenethylamine (b), ecgonine methyl ester (c), cocaine (d), cocaethylene (e).

had been passed. The LOD for the five compounds ranged from 0.50ng (EME) to 0.05ng (COC and CE). AEME and 3-ethoxy-4-methoxyphenethylamine had an intermediate LOD of 0.1ng.

Freeze/Thaw Method and Blind Study

This method produced results in six known cocaine-positive hair samples in the form of a peak for cocaine at the correct retention time which was visible and discernible from other peaks in the chromatogram. This peak was present in positive samples only, showing no contamination or carry-over was occurring. Six known cocaine-negative hair samples taken from different individuals did not yield this peak. Cocaine was the only compound of the five investigated that was present. However, all four ions for cocaine were found when an extracted ion profile was performed on the resulting chromatogram.

Twelve blind samples from Willow Laboratories were analyzed using the freeze/thaw method and GC/MS using the splitless SIM method. Six of the twelve blind samples tested positive for cocaine. All of the results concurred with those found by Willow Laboratories when the samples were analyzed by Willow Laboratories using their methodology.

In the blind samples determined to be positive for the presence of cociane, all four ions for cocaine were found. Like the known samples tested, cocaethylene, anhydroecgonine methyl ester, ecgonine methyl ester, and 3-ethoxy-4-methoxyphenethylamine were again absent from the positive samples analyzed in the blind testing. Resulting total ion chromatograms from this testing are found in Figure 3. Figure 3a involves the comparison of known cocaine-positive and cocaine-negative hair samples as well as one positive blind sample. The cocaine peak is found in both the blind sample and the positive control, but not in the negative control sample (the boxed area of Figure 3a). Figures 5b shows an extracted ion profile from this peak from the blind sample using the SIM method.

Minimum Amount of Hair Necessary for the Freeze/Thaw Method

It was found that cocaine was detected with as little as 10mg of hair in cocaine-positive samples, but that the abundance of cocaine was much smaller than when 20 and 30mg were used in the analysis as illustrated in the total ion chromatogram pictured in Figure 4. Thus, though it is possible to detect cocaine with 10mg of hair, it is recommended to use 20-30mg of hair for analysis to ensure that smaller concentrations of cocaine may still be detected using this method.

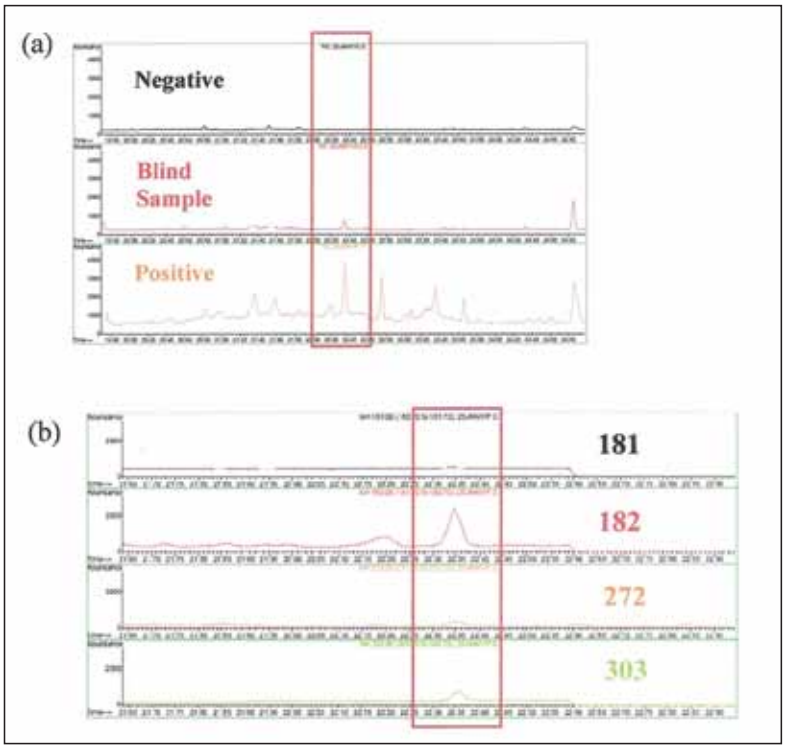

Figure 3. (a) TIC for a known cocaine-negative hair, known cocaine-positive hair, and a blind hair sample extracted using the freeze/thaw method. (b) EIP of the cocaine peak from the blind sample.

DISCUSSION

The freeze-thaw method of hair extraction used in this research is a fairly simple technique that allowed for the detection of cocaine in hair. When using GC/MS with this method, the cocaine peak which resulted was easily detected when compared to other surrounding peaks and was easily distinguished from peaks pertaining to the hair.

This peak was absent in negative samples and thus the occurrence of this peak at the correct retention time and the presence of the four ions chosen for cocaine when performing extracted ion profiling on the total ion chromatogram was indicative of cocaine being present in the sample. There was one instance where only three of the four ions were found in a positive sample. The fact that the peak occurred at the correct retention time as well as the molecular ion being present in strong abundance leads to the conclusion that the peak is due to cocaine being present in the sample.

The results of this research not only show proof of concept for this extraction technique, but also have significant implications for the detection of cocaine and possibly other drugs in hair. As of now, extraction methods such as enzyme-linked immunosorbent assay (ELISA) are employed. ELISA methodologies are very time consuming and sample preparation intensive if not automated and often require multiple hairs to be employed. The current study provides an alternative to ELISA and other such methods, allowing for the detection of cocaine in hair via a considerably less time consuming and labor intensive method. In a laboratory setting, the extraction may be performed even quicker utilizing liquid nitrogen. The freeze/thaw method does not require rigorous sample preparation and the analysis process is straightforward and easy to interpret. In addition, the analysis method is benefited by the fact that derivation of analytes is not needed.

There are future considerations which warrant further study. For example, as aforementioned the run time for the GC/MS could be decreased by adjusting certain parameters such as flow rate and other types of columns can be examined as well. This would further decrease the total analysis time giving an even more time efficient method.

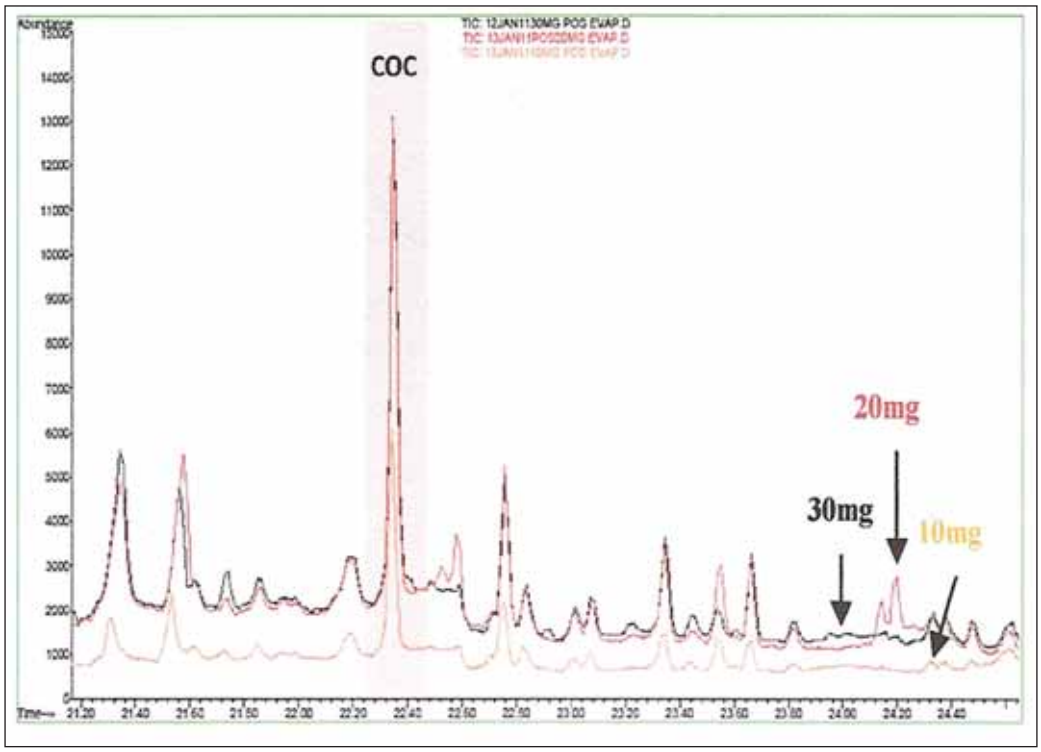

Figure 4. TIC illustrating the difference between 30, 20, and 10mg of known cocaine-positive hair when extracted with the freeze/thaw method.

Different solvents could be investigated to determine if the extraction could be improved. The number of freeze/thaw cycles could be investigated to determine if five cycles are necessary, or if analysis time could be decreased further by diminishing the number of cycles used.

Quantitative analysis and segmental hair analysis, which offers the possibility of estimating the time frame when an individual used a particular substance, could also be explored. This method could also be extended to other drugs, both illicit and pharmaceutical, and it would be interesting to investigate this method with hair sources other than head hair. Not everyone will have a source of head hair for collection and analysis, and thus beard, leg, chest, or pubic hair could be investigated using this method. In addition, the benefit of derivation of analytes should be explored to determine if ions from cocaine metabolites can be detected. It may also be important to determine if this method has any effect on mitochondrial DNA testing of hair. If not, DNA analysis could be performed on the samples after subjecting them to the freeze/thaw method of extraction. Many of these aspects could not be explored in this study due to a lack of availability of cocaine-positive hairs from the testing laboratory as well as commercially available cocaine-positive hair standards.

Overall, the freeze/thaw method appear to be viable for the detection of cocaine in hair which is applicable to work performed in forensic laboratories. It offers the advantage of simplicity and efficiency for the detection of analytes in hair and opens the door for further research in this area.

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