

Consequences of Decontamination Procedures in Forensic Hair Analysis Using Metal-Assisted Secondary Ion Mass Spectrometry Analysis

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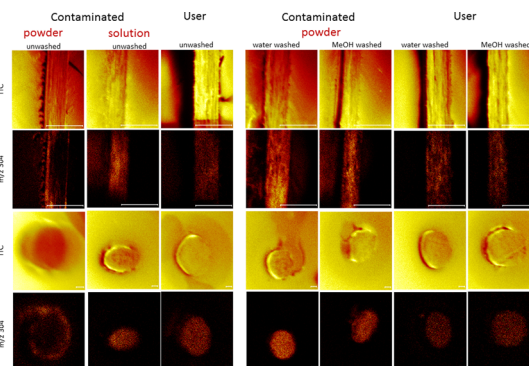
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ABSTRACT: Today, hair testing is considered to be the standard method for the detection of chronic drug abuse. Nevertheless, the differentiation between systemic exposure and external contamination remains a major challenge in the forensic interpretation of hair analysis. Nowadays, it is still impossible to directly show the difference between external contamination and use-related incorporation. Although the effects of washing procedures on the distribution of (incorporated) drugs in hair remain unknown, these decontamination procedures prior to hair analysis are considered to be indispensable in order to exclude external contamination. However, insights into the effect of decontamination protocols on levels and distribution of drugs incorporated in hair are essential to draw the correct forensic conclusions from hair analysis; we studied the consequences of these procedures on the spatial distribution of cocaine in hair using imaging mass spectrometry. Additionally, using metal-assisted secondary ion mass spectrometry, we are the first to directly show the difference between cocaine-contaminated and user hair without any prior washing procedure.



Hair testing is a powerful tool that is routinely used for the detection of drugs of abuse. The analysis of hair is highly advantageous as it can provide prolonged detectability compared to biological fluids. Moreover, chronological information about drug intake based on the average growth of hair can be obtained. Nevertheless, one of the drawbacks of drug testing in hair is the limited ability to distinguish between active users and contaminated subjects.¹ Recently, it was found that even pubic hair might produce false positive results due to external contamination.² To minimize the possibility of external contamination causing misinterpretation, several methods have been proposed. One of these methods is the adoption of a cutoff value. For cocaine, the proposed cutoff value that has been suggested is 0.5 ng/mg.³ Nonetheless, according to the most recent recommendations of the Society of Hair Testing, the simple use of cutoff levels is insufficient because external contamination can occur at any level.⁴ Moreover, it is stated that standardized wash procedures that will effectively remove any trace of external contamination without actively removing the drugs incorporated into the hair are not currently available, and as such the possible role of external contamination must be

considered when interpreting hair-testing findings. However, several approaches have been described to discriminate between external contamination and drugs incorporated through ingestion. Another proposed method is the detection of the relevant metabolites.⁵ However, this is only true for particular drugs in some circumstances. Even if certain metabolites, such as cocaethylene, are sufficiently specific to indicate the certainty of use, others such as benzoylecgonine may also occur as contaminant.⁶ Therefore, even the detection of metabolites is insufficient to exclude external contamination in all cases. Another frequently discussed solution is the decontamination of hair samples by washing the hair prior to analysis. Washing hair samples prior to analysis serves two purposes: the first is to remove hair products, sweat, sebum, and other surface materials that may interfere with the analysis or that may reduce extraction recovery, and the second is to remove deposited drug from sources such as sweat and

environmental contamination. Various authors have proposed different decontamination procedures like washing with methanol,⁷ dichloromethane,⁸ isopropanol,⁹ isopropanol and phosphate buffer,¹ ethanol and phosphate buffer,^{10,11} etc. Today, the general recommendation of the Society of Hair Testing is a decontamination strategy that includes an initial organic solvent, to remove oils, followed by aqueous washes.¹² Notwithstanding this general recommendation, it is shown in several papers that, even using the most sophisticated decontamination procedures, significant concentrations of cocaine may still be detected.^{13–15} Modern hair analysis also uses hydroxymetabolites of cocaine for differentiation of intake from contamination.⁵ Those authors found that “cocaethylene, norcocaine, and hydroxycocaine metabolites were only present in COC (cocaine) users’ hair and not in drug chemists’ hair”. Nevertheless, there are case reports in which no hydroxymetabolites are reported although a history of cocaine use is known. Thus, false negatives can be expected. Finally, to considerably help the interpretation of the results, an additional step involving the comparison of the drug level in the wash residue compared to the level in hair was proposed.¹⁶ Even with this additional step, the likelihood of external contamination confounding the interpretation of hair testing results is only reduced, but not completely excluded.

New techniques such as matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) are explored to detect drugs after pulverizing and extracting the hair.¹⁷ Recently, mass spectrometry imaging (MSI) techniques were introduced into the field. Using these techniques, multiple drug analysis on the same single hair can be carried out. Porta et al. already demonstrated the analysis of cocaine in a single intact user’s hair using MALDI-triple quadrupole linear ion trap.¹⁸ Musshoff et al. presented the determination of cocaine and cannabinoids in a single hair using a MALDI-LTQ Orbitrap XL instrument, confirming the conclusion of Porta et al. that MALDI-MS is applicable for the determination of drugs and pharmaceuticals in hair.¹⁹ Notwithstanding this, a few articles have been published using imaging mass spectrometry to make a distinction between external contamination and intake,^{20,21} until to date it remained impossible to directly and indisputably demonstrate the difference between external contamination and drug use. Furthermore, despite the fact that decontamination protocols are considered to be indispensable in the indirect discrimination between external contamination and drug abuse, the exact consequences of these decontamination procedures on the distribution of (incorporated) drugs are unknown. In this paper, we show these consequences on the distribution of cocaine in hair, applying different washing procedures, using imaging mass spectrometry techniques such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and metal-assisted secondary ion mass spectrometry (MetA-SIMS). Using the latter technique, we are able to directly show the difference between cocaine-contaminated and user hair without any prior decontamination step.

■ MATERIALS AND METHODS

Reagents and Materials. Alpha-cyano-4-hydroxycinnamic acid (CHCA), carboxymethylcellulose, and dichloromethane (DCM) were purchased from Sigma-Aldrich (Germany). Acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), hexane, and trifluoroacetic acid (TFA) were purchased from Biosolve (The Netherlands). Cocaine base standard was purchased from Lipomed (Arlesheim, Switzerland). Cocaine-

HCl was purchased as a powder from a hospital pharmacy. Cocaine-d3, benzoylecgonine-d8, and methylecgonine-d3 were purchased from Cerilliant (U.S.A.).

A 1 mg/mL cocaine·HCl stock solution was made in phosphate buffer, pH 6.0. Cocaine base solution of 1 mg/mL was prepared in methanol. Contamination solutions were freshly prepared. All contamination experiments were performed with base as well as hydrochloride salt.

Sample Preparation and Analysis. Prior to imaging, blank and user hairs from volunteers were contaminated by rubbing cocaine salt powder on the hair for a few minutes or by soaking them for 5 min or 5 h in a 1 mg/mL solution of cocaine base or HCl salt. Washing was performed by shaking the hair for 1 min in the described washing solution. All hairs were air-dried after washing and before cutting, embedding, or mounting them. The effects of different washing solvents (methanol–water, dichloromethane, isopropanol, acetonitrile, and dichloromethane/water alternately) were tested on contaminated as well as cocaine user hair. Washing solvents were also tested on hairs from different cocaine users. Blank (negative) hairs were tested using the same method.

For analysis with MALDI-MS, intact hairs were mounted on a glass slide using double-sided tape. Samples were coated with a 10 mg/mL solution of CHCA in 70:30 acetonitrile/water with 0.2% TFA using the Bruker ImagePrep (Bruker Daltonics, Bremen, Germany). A Waters MALDI HDMS SYNAPT mass spectrometer (Waters Corporation, Manchester, U.K.) equipped with a 200 Hz, 355 nm Nd:YAG laser with a spot diameter of 150 μ m was used to acquire mass spectra and images. Prior to MALDI-MSI analysis, the samples were optically scanned using a flatbed scanner to produce a digital image for future reference. This image was then imported into the MALDI imaging pattern creator software (Waters Corporation) to define the region to be imaged. The instrument was calibrated prior to analysis by using a standard mixture of polyethylene glycol. The instrument was operated in V-mode and positive ion mode; the data was acquired in the mass range m/z 50–350. The ion of interest was fragmented by collision-induced dissociation (CID), and the fragment transition monitored for cocaine was m/z 304 \rightarrow 182, at a collision energy of 10 eV.

For analysis with metal-assisted secondary ion mass spectrometry (MetA-SIMS), hairs were embedded in a 2% w/v solution of carboxymethylcellulose (CMC) in water and immediately snap-frozen using liquid nitrogen. The embedded hair samples were then sectioned at a thickness of 12 μ m using a Microm HM550 cryo-microtome (Microm International, Walldorf, Germany). The cross sections were thaw-mounted onto clean indium tin oxide (ITO) slides (Delta Technologies, U.S.A.). Longitudinal sections of hair samples were accomplished using the cutting apparatus as described.²³ Prior to analysis, hair samples were gold-coated using a Quorum Technologies SC7640 sputter coater (New Haven, UK) equipped with a FT7607 quartz crystal microbalance stage and FT690 film thickness monitor to deposit a 1 nm thick gold layer. Data were then acquired using a Physical Electronics TRIFT II TOF-SIMS (Physical Electronics, U.S.A.) equipped with an Au liquid metal gun tuned for 22 keV Au⁺ primary ions. Data were analyzed and visualized using WinCadence software version 4.4.0.17 (Physical Electronics). The instrumental mass range is m/z 0–1850.

Conventional LC-MS/MS analysis was carried out using a Waters Acquity UPLC system with a triple quadrupole mass spectrometer, Quatro Premier XE, Micromass/Waters. A

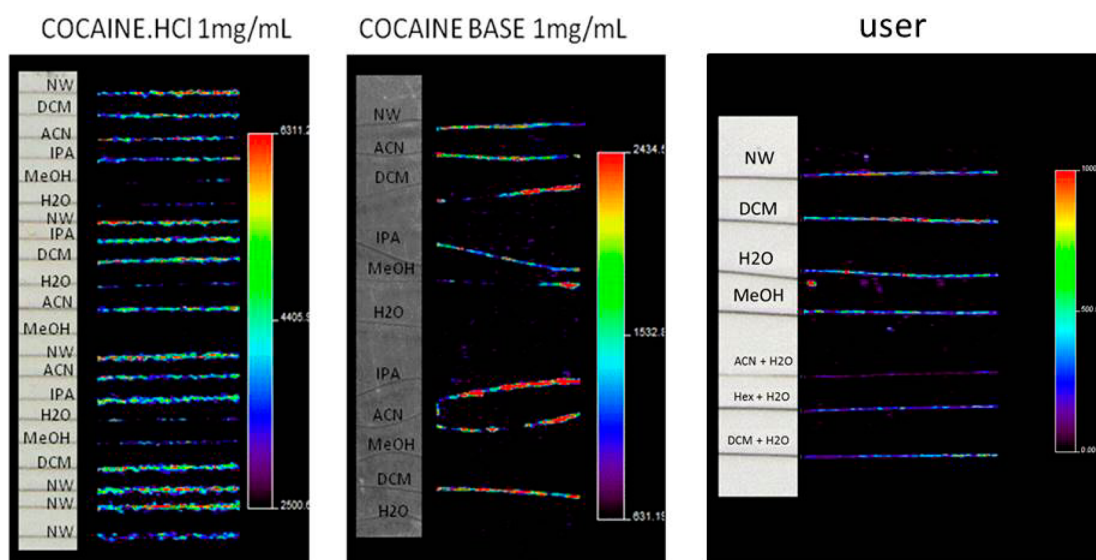


Figure 1. MALDI-MS/MS images of the cocaine precursor ion (m/z 304 \rightarrow 182) of (Left) cocaine-HCl contaminated hair, (Middle) cocaine base contaminated hair, and (Right) cocaine users' hair. Hairs were 1 min washed with indicated medium. NW, not washed; DCM, dichloromethane; ACN, acetonitrile; IPA, isopropanol; MeOH, methanol; H₂O, water; Hex, hexane. Images show a significant decrease in cocaine after washing with methanol and/or water.

Precolumn UPLC Waters vanguard, BEH C18, 1.7 μ m, 5 \times 2.1 mm i.d., was used together with an UPLC Waters Acquity BEH C18 100 \times 2.1 mm i.d., 1.7 μ m analytical column. The sample injection volume was 3 μ L. The ultra-performance liquid chromatograph (UPLC) was operated with a gradient 5:95, A/B mobile phase (A = methanol/B = 10 mM ammonium bicarbonate, pH 10.0), to 95:5, A/B at 6 min holding until 8 min, and back to 5:95, A/B at 8.01 min. The total runtime was 10 min with a flow rate of 0.500 mL/min. Ionization mode was ESI+, and collision energy was 20 eV for cocaine and 18 eV for benzoylecgonine and methylecgonine. Ion source temperature was 130 $^{\circ}$ C, desolvation temperature was 500 $^{\circ}$ C, and desolvation gas flow was 1000 L/h. The following pairs of ions were monitored with the following values of m/z : 304.22/182.10 and 304.22/104.90 for cocaine; 290.22/168.10 and 290.22/105.00 for benzoylecgonine; and 200.12/182.10 and 200.12/82.00 for methylecgonine. Cocaine.d3 was monitored with 307.22/185.1 and 307.22/105.00; benzoylecgonine.d8 was monitored with 298.28/171.10 and 298.28/110.00; and methylecgonine.d3 was monitored with 203.13/185.10 and 203.13/119.00.

RESULTS AND DISCUSSION

Comparison of Different Washing Protocols Using MALDI-MS/MS and Meta-SIMS. The effects of different routinely used washing solvents were tested on contaminated as well as cocaine user hair. All hairs were separately washed in the described washing solution for 1 min. MALDI-MS/MS images (Figure 1) show the distribution and concentration of cocaine (m/z 304 \rightarrow 182) along the intact hair after extracting it by the CHCA matrix solution. For cocaine-contaminated hair, no differences were seen between hair contaminated with cocaine salt and base. In both cases, washing with water or methanol produced a significant decrease in cocaine concentration. All other solvents were inefficient in removing the contamination from the hair, making them unsuitable as a washing solvent prior to hair analysis. Washing solvents were also tested on hairs from different cocaine users (Figure 1C). Here, none of

the solvents caused a significant decrease in cocaine concentration. An initial wash with organic solvent (acetonitrile, methanol, or dichloromethane) followed by a water wash, as recommended by the Society of Hair Testing,¹² did not have a significant effect as compared to the unwashed hair. Because washing procedures are thought to remove primarily external contamination and incorporated cocaine is expected to remain in the hair, one does not expect to see a significant decrease in cocaine concentration in user hair after washing. Taking into account that the MALDI matrix solution is expected to extract cocaine out of the hair, it is not surprising that cocaine is still found in the MALDI image of user hair while the majority of cocaine in the externally contaminated hair is removed by water and methanol. Consequently, the differences in the effect of the methanol and water washes between contaminated and user hair are most probably due to the difference in localization of the cocaine: the remaining cocaine in the user hair image was most probably incorporated cocaine. Therefore, based on the shown MALDI results, only water or methanol might be considered as a good washing solvent because it only removes cocaine on external contaminated hair and but not in user hair.

Nevertheless, one should always be aware that MALDI relies on the extraction of the compound of interest. It has already been shown with tissue samples that reapplying matrix and repeating MALDI-MS analysis gives additional information on most compounds. This indicates that the extraction efficiency by the MALDI matrix of most compounds is not 100%.²² Because extraction efficiency is limited in tissue sections and considering the structure of an intact hair, which consists of rather impermeable external cuticle scales, the extraction efficiency of the MALDI matrix on intact hair is probably poor. For this purpose, we need to have a more detailed look into what is taking place inside the hair. To this end, a hair-sectioning technique combined with a high spatial resolution imaging technique is required.

To have a closer look into the effect of water and methanol washes on the cocaine distribution in hair, first intact contaminated hairs were imaged using a high spatial resolution

Meta-SIMS technique. Figure 2 shows that cocaine contamination in unwashed hair mainly concentrates along the cuticle

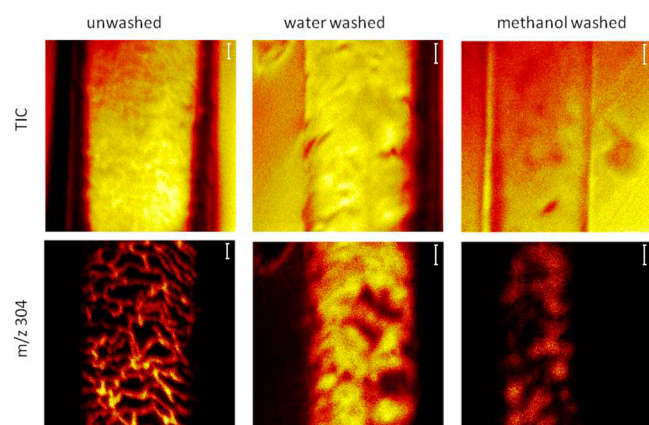


Figure 2. Meta-SIMS images of **uncut** cocaine-HCl-contaminated hair. Total ion current (TIC) and cocaine (m/z 304) are shown. Hair indicated as “washed” was washed for 1 min with water or methanol. Images show the effect of washing solvents on the distribution and concentration of cocaine. Cocaine contamination in unwashed hair mainly concentrates along the cuticle edges, while water spreads the cocaine over the whole cuticle. Methanol mainly decreases the cocaine concentration. Scale indicates 10 μ m.

edges (the cuticle structure is clearly visible on the image), while water seems to spread the cocaine along the whole cuticle. Methanol mainly seems to spread the cocaine and decreases its concentration. The shown spreading of external cocaine, induced by water, might also explain the difference after water washes between the contaminated versus user hair in the MALDI images. The cocaine incorporated in user hair is not amenable to this spreading and is thus still visible in the MALDI images, while cocaine on the cuticle might be spread and is thus strongly diluted when covering the hair with water-based matrix solution.

■ META-SIMS ANALYSIS TO REVEAL WHAT IS TAKING PLACE INSIDE THE HAIR

Because MALDI-MS imaging is based on the extraction of the compound of interest and one will never be completely sure whether the detected compounds were originating from external contamination or extracted out of the hair, there is a need to have a closer look inside the hair. Therefore, we developed a cross-sectioning method as well as a longitudinal cutting method,²³ which can be used as a sample preparation for high spatial resolution imaging techniques such as Meta-SIMS. As shown in Figure 3, there is a clear difference in cocaine distribution in unwashed powder-contaminated versus user hair: longitudinal as well as cross sections show a

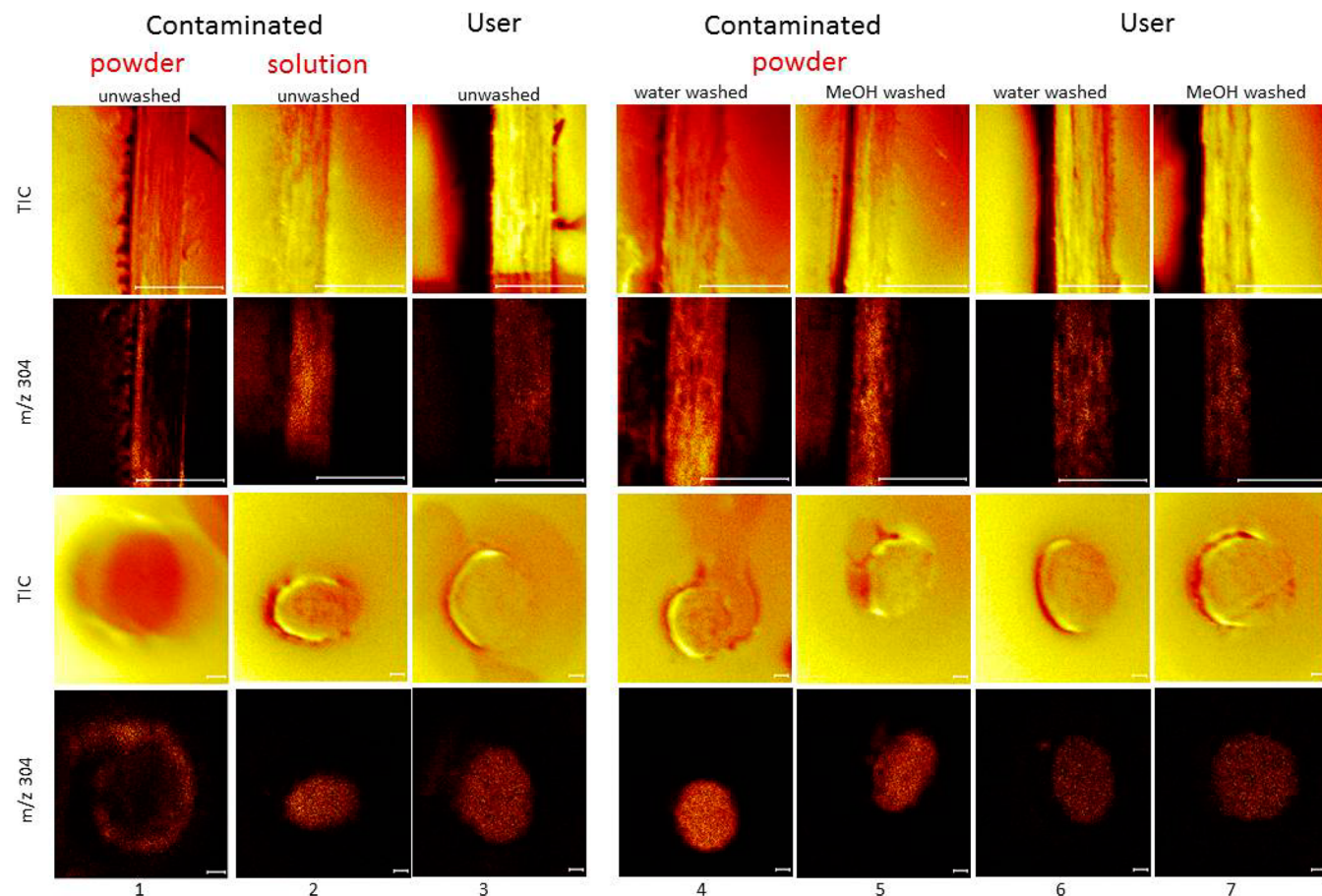


Figure 3. Meta-SIMS images of **longitudinal** (first and second rows) and **cross-sectioned** (third and fourth rows) cocaine powder- and solution-contaminated (columns 1, 2, 4, and 5) and user hair (columns 3, 6, and 7). Total ion current (TIC) and cocaine (m/z 304) are shown. A clear difference can be seen in the longitudinal as well as the cross section of powder-contaminated versus solution-contaminated or users' hair. After washes with water or methanol, the differences fade away due to the effect of the solvents on the distribution and concentration of the washing solvents. Scale on longitudinal sections indicates 100 μ m; scale on cross sections indicates 10 μ m.

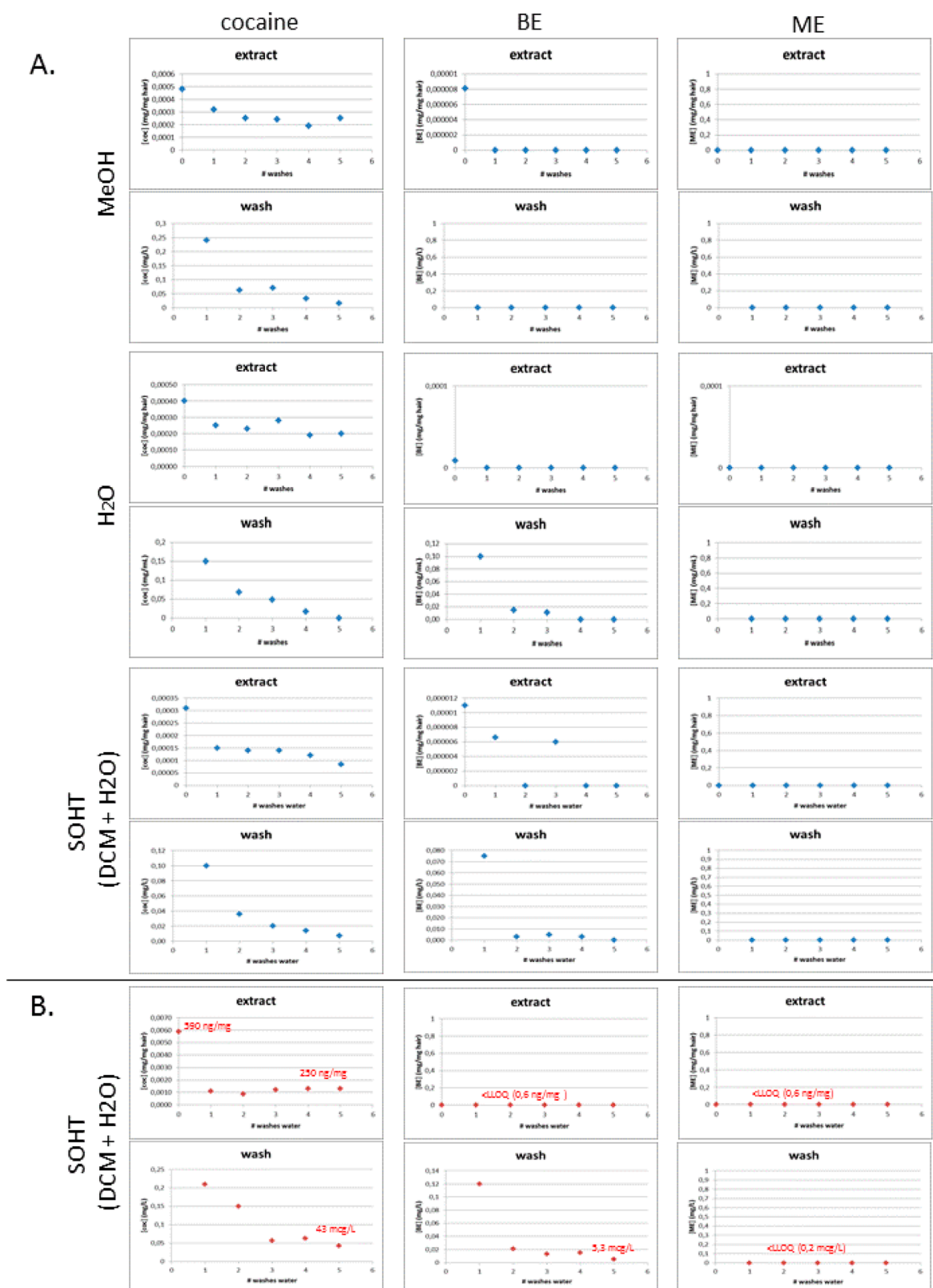


Figure 4. (A) LC-MS/MS quantification results of **cocaine solution-contaminated** hairs after different decontamination protocols and “conventional” extraction procedure. The cocaine, benzoylecgonine (BE), and methylecgonine (ME) concentrations in the different consecutive washing solutions and the hair extract are shown. (B) The same results as indicated in (A) for **cocaine powder-contaminated** hairs.

concentration of cocaine in the cuticle of contaminated hair, while it is detected in cuticle, cortex, and medulla in users’ hair. Surprisingly, washing procedures clearly influence this distribution. Water as well as methanol apparently induces a

migration of external cocaine into the cortex and medulla of contaminated hair, making it indistinguishable from cocaine user hair. This can be explained by the previously postulated mechanism that the scales on the cuticle rise in the presence of

moisture, which allows readier access to the interior and provides a vehicle, in this case containing cocaine, for diffusion.²⁴ This theory also explains the difference between unwashed powder-contaminated and solution-contaminated hair. Indeed, when hair is contaminated with cocaine solution, we noticed that cocaine can be detected inside the hair (cortex and medulla).

Looking back to the MALDI results, we can conclude that the previously stated question about extraction of the MALDI matrix is indeed relevant. Because MetA-SIMS analysis proved that external cocaine is, at least partly, incorporated into the hair when using water or methanol as solvent, we expect cocaine to remain visible in Figure 1A and B. Nevertheless, in both cases, (almost) no cocaine was detected, indicating that the extraction efficiency and consequently the detection limit of MALDI is inefficient for forensic hair analysis. For the cocaine user image (Figure 1C), we would expect cocaine to be visible in all (washed) hairs, which was indeed the case. This indicates that incorporation of cocaine at a molecular level due to use is different in nature than incorporation of external contamination during washing. Indeed, the incorporation of cocaine is fixated uniformly across the hair and does not seem to be distributed upon washing.

Comparison with "Conventional" Extraction Strategy Combined with LC-MS/MS Analysis. The next question that arises is what the conclusion would be when the cocaine-contaminated hairs would be analyzed the "conventional" way, using extraction and LC-MS/MS detection. Therefore, user hair and solution- and powder-contaminated hair were washed up to 5 times with methanol, water, or water and dichloromethane (DCM) according to the Society of Hair Testing (SOHT) decontamination recommendations. Afterward, conventional extraction was carried out by pulverizing 10.0 mg of hair and extracting it with methanol at 37 °C. The concentration of cocaine in the user hair, known to be a chronic cocaine user, was determined using MeOH extraction after grinding and LC-MS/MS analysis. The cocaine concentration was found to be 250 ng/mg of hair. Also, benzoylecgonine (BE), norcocaine, cocaethylene (CE), and hydroxycocaine were detected. The CE/COC ratio is 0.2%, and the BE/COC ratio is 0.51. Figure 4 shows the cocaine, BE, and methylecgonine (ME) concentrations in the consecutive washes as well as in the corresponding extracts. Note that, after 2 washes, the cocaine concentration in the hair extract does not decrease significantly anymore. Because there is no significant decrease in cocaine concentration in the consecutive washes, international guidelines advise to start extracting the hair. Nevertheless, these hair extracts are still considered to be positive when the international accepted cutoff level of 5 ng/10 mg of hair is taken into consideration. Benzoylecgonine and methylecgonine were detected in trace amounts below the lower limit of quantification (0.2 ng/mL of wash and 0.6 ng/mg of hair for BE and ME). This indicates that trace levels of BE and ME can be formed from external sources. Thus, the detection of these compounds cannot be used as proof for body passage nor in the differentiation between contaminated and user hair. These results show that, taking into consideration the current international accepted hair analysis decontamination and extraction protocols, contaminated hair might be considered as positive and thus misinterpreted as cocaine user's hair.

■ CONCLUSION

The results presented here question the current internationally accepted strategy of decontamination of hair prior to analysis. The most recent SOHT guidelines stated that it is generally accepted that organic solvent such as dichloromethane or acetone will remove only surface contamination.⁴ Nevertheless, we showed that there is still cocaine detectable after these washes using imaging mass spectrometry. Moreover, it is highly suspected that these solvents do wash in external contamination because cocaine as well as BE and ME are detected in extracts from contaminated hair that was washed using dichloromethane. MetA-SIMS images show that decontamination solvents, which are currently used to wash off high concentrations of cocaine on the hair, at the same time promote the external cocaine to migrate into the hair. This "washed-in" cocaine might be considered as incorporated since it is shown to possibly reach above the international cutoff level. We are aware of the fact that the cocaine concentration on the contaminated hair tested is higher than in real-case scenarios. Nevertheless, these experiments prove the possible redistribution of cocaine to the inside of the hair. It can be expected that the mechanism remains the same, regardless of the concentration. The amount of redistributed cocaine might differ depending on the contaminating amount of cocaine. Further research regarding the amount of redistributed cocaine in relation to the external contaminating amount is necessary.

Proposed cutoff levels by SOHT are shown not to be sufficient in distinguishing between contamination or use. The proposed cutoff value of 0.05 for the BE/cocaine ratio might be, in most cases, a more stringent criteria for differentiating between contamination and use. Indeed, in our contaminated hair samples, most ratios were indeed below this cutoff. Some publications, however, found BE/cocaine ratios in user's hair below this cutoff.²⁵ Consequently, using this cutoff ratio will induce false negative results.

In conclusion, the so-called and currently used decontamination process of hair will influence the interpretation of forensic hair analysis results as initial external contaminated hair might be considered as user's hair. The high spatial resolution technique MetA-SIMS is used for the first time to investigate the spatial distribution of a compound in hair. Moreover, this is the first research that is able to directly show the difference between drug-contaminated and user's hair and furthermore questions the current and internationally accepted forensic hair analysis protocols. Therefore, one should be very cautious to use any kind of washing procedure to rule out external contamination. Because this research provides new insight into drug incorporation in hair, it will form the basis for new international hair analysis criteria and analysis protocols.

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Notes

The authors declare no competing financial interest.

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