Hydrogen peroxide reactions on cocaine in hair using imaging mass spectrometry

KEYWORDS

Imaging mass spectrometry, forensic hair testing, hydrogen peroxide, cocaine

ABSTRACT

Today, forensic hair analysis is considered to be a standard method for identifying chronic drug users since information about drug use stored and located in hair can cover several months to even years. When interpreting these results, one should be aware of all kind of pitfalls. External factors such as bleaching might influence the analytical result. Although the effect of hydrogen peroxide on cocaine in a solution was described before, it was never investigated whether the described reaction products (ecgonine methylester, benzoylecgonine, hydroxynorcocaine and dihydroxycocaine) are indeed found on contaminated or user hair. Since it is of great importance in forensic hair analysis to know whether cocaine and/or reaction products are detectable in hair after bleaching, matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) was used to study the effect of hydrogen peroxide treatment on incorporated cocaine in hairs. Cocaine oxidation products were identified in a solution based on MS/MS spectra and spatial distribution of these products in hair was explored using MALDI TOF-MS. All images were accomplished by spraying α-Cyano-4-hydroxycinnamic acid (CHCA) as a MALDI-matrix. Images revealed a loss of detectability of cocaine and its reaction products in hairs already after a short bleaching period. Since all compounds of interest are found in the hydrogen peroxide and wash solution, these findings indicate that all evidence of cocaine use might be lost after a hair bleaching treatment. Therefore, forensic toxicologists should take into consideration whether hair samples were bleached before making any conclusions from hair analysis results.

INTRODUCTION

Forensic hair analysis is considered to be a standard method for identifying chronic drug (ab)users since it can give information about the time course of the substance use. Nevertheless, when interpreting these results, one should be aware of all kind of pitfalls in hair analysis. One important issue of concern is the change in drug concentrations induced by cosmetic treatment of hair. Numerous articles already described the possible influence of cosmetic treatment on hair analysis [1-9]. Most of these studies, using conventional pulverizing and extraction methods, reveal a decrease in detectability of the compound of interest. Unfortunately, the reason for this decrease was hardly ever studied. Only Tanaka *et al.* had a closer look into cocaine degradation by studying possible reaction products mixing cocaine with hydrogen peroxide in a solution. Although benzoylecgonine is generally considered to be one of the important compounds to improve the discrimination of use from external contamination, it was described in Tanaka's paper to be one of the six degradation products [10, 11]. Notwithstanding this, evidence that the described reaction products are indeed formed and remain in bleached cocaine user hair, was never shown. Nevertheless, these reaction products may be of great interest in the analysis and interpretation of forensic hair samples since they might shed new light on the use of benzoylecgonine as a discriminator between use and contamination. Other possible discriminators such as cocaethylene and norcocaine were already described in cocaine contaminated hair, making them of very limited importance as (ab)use indicators [12]. Therefore, other described reaction products as (di)hydroxycocaine might be the only evidence left for (chronic) drug use.

Over the past few years, mass spectrometry imaging (MSI) has become of interest in forensic toxicology field as it does not require time consuming steps such as pulverization, digestion and extraction are not required [7, 13-19]. Another great advantage of MSI is the fact that the sample is not destroyed during analysis and thus several tests can be carried out on the same sample. However, one should keep in mind the lack of statistical representation since it was recently described that analysis of single hairs can lead to misinterpretation due to different growth rated and particularly the phenomenon of different growth phases [17]. Nevertheless, MALDI-MSI was already described as a feasible and promising technique in the analysis of one single hair to detect cocaine consumption using a straightforward sample preparation and allowing a spatial resolution of 1 mm, giving chronological information about consumption. A limit of detection of 0.1 ng/mg was described for cocaine [20]. Although MSI might also be a great tool for studying external influences on drugs in hair, the effect of cosmetic treatment on incorporated drugs was never studied with MSI.

In this paper, we present a mass spectrometric imaging approach to study whether cocaine and/or reaction products are detectable in cocaine contaminated and user hair after bleaching. Insights into the reaction products and their possible incorporation in hair will be of great importance in the understanding and interpretation of forensic analytical hair results.

EXPERIMENTAL

Chemicals

Cocaine base and cocaine.d3 standard was purchased from Lipomed (Arlesheim, Switzerland) as a 1 mg/mL solution in methanol. Cocaine.HCl was purchased as powder from a hospital pharmacy. A 1mg/ml cocaine.HCl stock solution was made in phosphate buffer pH 6.0. All stock solutions were kept at -20°C. All contamination experiments were performed with base as well as hydrochloride salt showing no differences. Hydrogen peroxide stock solution (Sigma Aldrich, Schnelldorf, Germany) 30 wt. % in H_2O was used in reaction product experiments and commercially available hair bleaching product Corona oxycream (hydrogen peroxide 9%) in hair bleaching experiments. α-Cyano-4-hydroxycinnamic acid (CHCA, Sigma Aldrich, Schnelldorf, Germany) was sprayed on all samples as matrix substance for MALDI-MS experiments in a 10 mg/mL solution of methanol/water (70/30 (v/v)) with 0,1% trifluoroacetic acid (TFA). Methanol, water and TFA were purchased from Biosolve (Valkenswaard, The Netherlands).

Protocols and equipment

The reaction product timeline was created as follows: a 1/1 (v/v) mixture of 100 µg/mL cocaine and hydrogen peroxide 30% was made and kept at 37°C in an oven. Immediately before testing the solution, a 1/1 (v/v) mixture was made with cocaine.d3 (100 µg/ml) in CHCA matrix and 1 µL was spotted on the MALDI target plate. MALDI measurements were immediately carried out after spotting.

Brown blank hairs were collected from a volunteer and contaminated by soaking them for 5 minutes or 5 hours in a solution of 1 mg/mL cocaine.HCl in water. User hair was collected from volunteer cocaine users. These cut hairs were air dried for at least 30 minutes before bleaching them. Bleaching was carried out using commercially available Corona oxycream containing 9% hydrogen peroxide. Hairs were put in this cream for 5, 15 or 60 minutes. The indicated washed hairs (W) were immediately washed after bleaching for 20 seconds with water. After air drying for at least 30 minutes, hairs were mounted onto a glass slide using double sides tape and sprayed with CHCA matrix. Matrix (CHCA) deposition was performed with an ImagePrep (Bruker Daltonics,Bremen, Germany) or a Suncollect system (SunChrom GmbH, Friedrichsdorf, Germany).

Digital optical scans of all hair samples were obtained prior to MALDI-MS experiments using a 600 dots per inch desktop scanner. The resulting digital images were imported into the MALDI Imaging Pattern Creator software (Waters Corporation). Instrument calibration was performed using a standard calibration mixture of polyethylene glycol with an MW of 100–3,000 (Sigma-Aldrich). A MALDI-quadrupole time-of-flight SYNAPT HDMS system (Waters) operating with

a 200-Hz Nd:YAG laser was configured to acquire data in the positive V-reflectron mode. Reaction products on bleached hair was measured in positive as well as negative mode. Data were acquired at a raster size of 150 µm X 50 µm. Ion images were generated with Biomap 3.7.5.5 software (Novartis Pharma). MS/MS spectra were obtained at a collision energy of 20 eV.

Peroxide reaction product mass spectra were obtained using Ultraflex III MALDI TOF-MS (BrukerDaltonik GmbH, Bremen, Germany). The exact mass of m/z 344 was obtained by a 9.4T MALDI Fourier Transform Ion Cyclotron Resonance imaging mass spectrometer (FTICR).

RESULTS AND DISCUSSION

Since it still remained unclear if the previously described cocaine degradation [6] is not only taking place in a solution, but also on the hair, a first bleaching experiment on cut cocaine contaminated hair was carried out. In order to visualize the effect of hydrogen peroxide on cocaine on hair, 5 minutes and 5 hours cocaine contaminated hair was entirely put in commercially available lipophilic hydrogen peroxide (9%) cream during the indicated time (figure 1). MALDI-MS/MS images show the distribution of the cocaine product ion m/z 182. Surprisingly, a bleaching period of 5 minutes already shows a significant decrease in cocaine found in 5 minutes contaminated hairs. It was observed that 5 hours contaminated hairs need a longer bleaching time (15 minutes) in order to give a significant decrease of cocaine. The reason for this observation may be due to the higher incorporation rate of cocaine into the hair when contamination time is increased. Since incorporated cocaine is expected to be less sensitive to external influences, the time needed to react with this incorporated cocaine might be much longer. Remarkably, a 20 seconds wash with water partly removes cocaine without any bleaching. This removal is more distinct in 5 minute contaminated hair, showing that more superficial contamination is removed more easily by short washing procedures. Consequently, the remaining amount of cocaine in 5 hours contaminated hairs after a washing procedure can be considered as at least partly incorporated into the hair and thus gives a good idea about the effect of bleaching on incorporated cocaine.

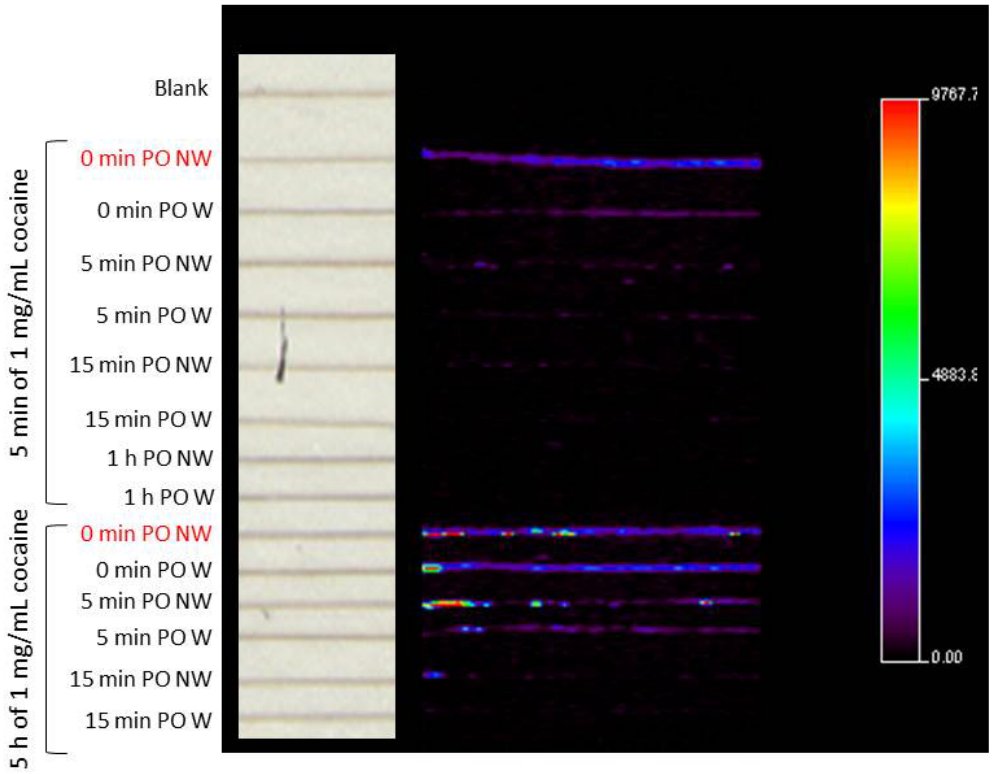

Figure 1: The effect of hydrogen peroxide (PO) 9% on 5 minutes and 5 hours cocaine ***contaminated*** *hair measured by MALDI-MS/MS showing the distribution of product ion m/z 182. Indicated washed hairs (W) were 20 seconds washed with water. NW indicates the non-washed hair.*

Although this first experiment was carried out on contaminated hair from the same person, one should keep in mind that different factors might influence the incorporation of drugs in hair even within the same person. In vitro binding studies already showed the influence of melanin and lipids on cocaine binding [21]. Also hair-shaft damage increases drug binding because drugs must first penetrate the cuticle, and this penetration is aided by the damage. Therefore, even hair from the same person might have different incorporation rates and might be influenced differently by the bleaching product. To rule out these intra-individual differences, the effect of hydrogen peroxide was tested on the same hair by bleaching only half of the same single contaminated hair (figure 2). These images reveal that the previous shown results in figure 1 are not caused by intra-individual differences since a clear difference in cocaine concentration is found between the bleached and non-bleached part of the same hair.

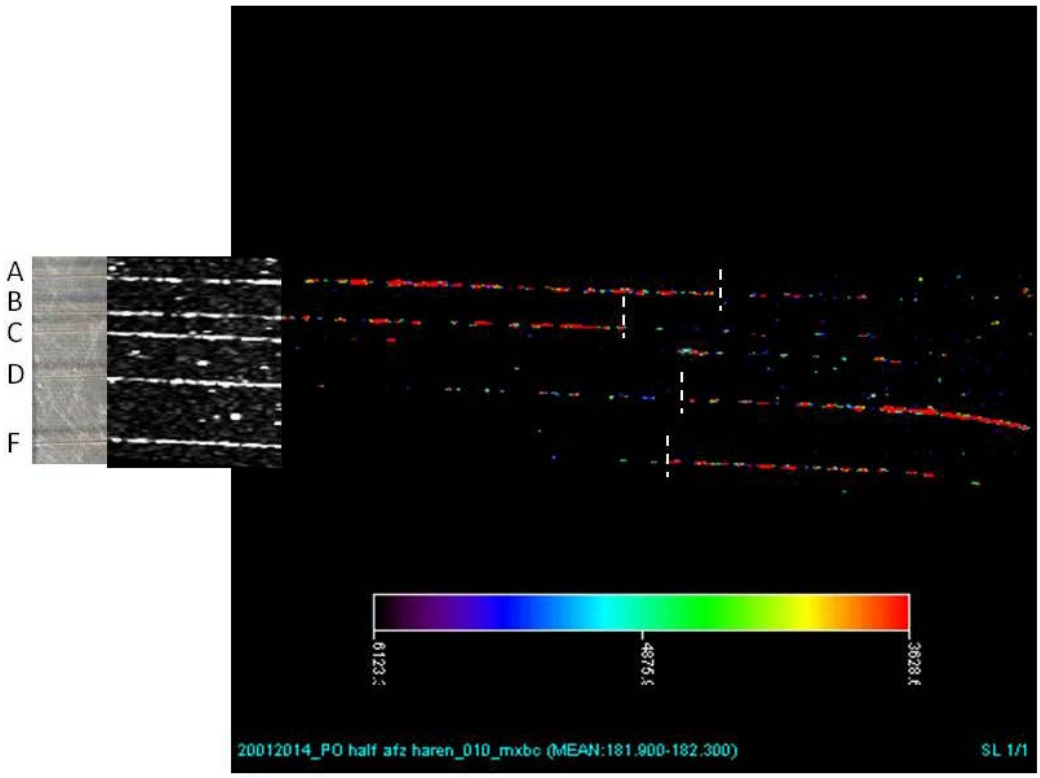

Figure 2: The effect of hydrogen peroxide 9% on 5 hours ***<u>contaminated</u>*** *hair measured by MALDI-MS/MS showing the distribution of m/z 182. On hairs A and B, the right side and on hairs D and F, the left side was bleached. Hair C was completely bleached using 9% hydrogen peroxide. The imaged hairs were not washed.*

Nevertheless, since external cocaine contamination might not reflect entirely the incorporation of cocaine into users hair, the effect of hydrogen peroxide on users hair was also studied (figure 3). Therefore, hairs from a known cocaine user were bleached for 15 minutes in Corona oxygen cream (9% hydrogen peroxide). The fragmentation ion m/z 182 was imaged in MS/MS mode. Concentration of m/z was compared in users hair with and without a 20s wash with water. No significant difference was found. Comparing m/z intensity of bleached and unbleached hair showed a clear reduction of the signal. Washing the hair after bleaching finally removed all evidence of any cocaine in the hair since no m/z 182 signal was found. These results show that also in users hair, all evidence of cocaine use is removed after a 15 minutes bleaching period with a commercial peroxide cream.

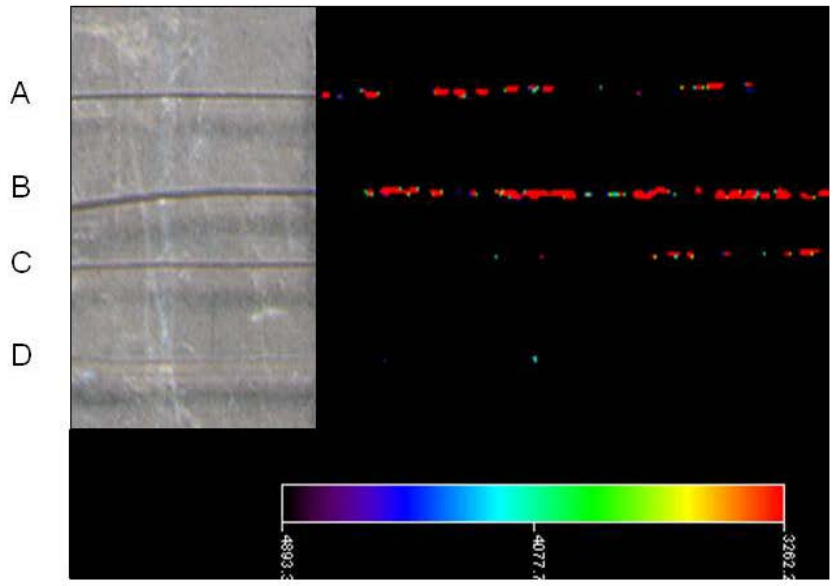

*Figure 3: The effect of hydrogen peroxide 9% on cocaine **user** hair measured by MALDI-MS/MS showing the distribution of m/z 182. **A.** is user hair unwashed, **B.** is user hair 20 s washed with water, **C.** is user hair unwashed and bleached 15 minutes with 9% hydrogen peroxide, **D.** is users hair 15 minutes bleached with 9% hydrogen peroxide and afterwards 20 s washed with water.*

All results shown indicate a significant loss of detectable cocaine in hair after a short bleaching period with a commercially available cream. A likely explanation is the chemical conversion of cocaine in the presence of hydrogen peroxide. As Tanaka *et al.* already described, several cocaine oxidation products can be formed. In order to identify all reaction products, a mixture of 1 mg/mL cocaine base and hydrogen peroxide (30% (v/v)) was kept at 37°C for 24 hours. Afterwards, MALDI-TOF MS(/MS) spectra were taken by spotting the solution on a target plate. In figure 4B, mass peaks in red indicate cocaine-peroxide reaction products after 24 hours. Most abundant reaction products were identified as ecgonine methylester (m/z 200), benzoylecgonine (m/z 290), hydroxynorcocaine (m/z 320) and dihydroxycocaine (m/z 336) based on their MS/MS spectrum (figure 5). These results are in agreement with the results of Tanaka *et al.* A potassium adduct of hydroxynorcocaine (m/z 344), which was not described before, was identified based on the exact mass measured on FTICR (exact m/z 344,0895). As shown in the reaction timeline (figure 5, top right), cocaine is only decreasing after a few hours. After 24 hours, there is still cocaine detected in the reaction solution which was kept at 37°C. Since reaction kinetics seem to be different in reaction solution compared to hair, these results indicate that studies in which reactions are tested in a solution do not necessarily simulate the reactivity of compounds incorporated into the hair. One should take into consideration that incorporated drugs in hair are bound to different types of proteins such as melanin, and thus a change in reaction kinetics as

compared to solutions can be expected. The different cocaine degradation in hair and solution as shown by our experiments shows that the effect of cosmetic care agents always should be tested on incorporated compounds into the hair instead of using solutions as simulating media since multiple factors may influence reaction kinetics.

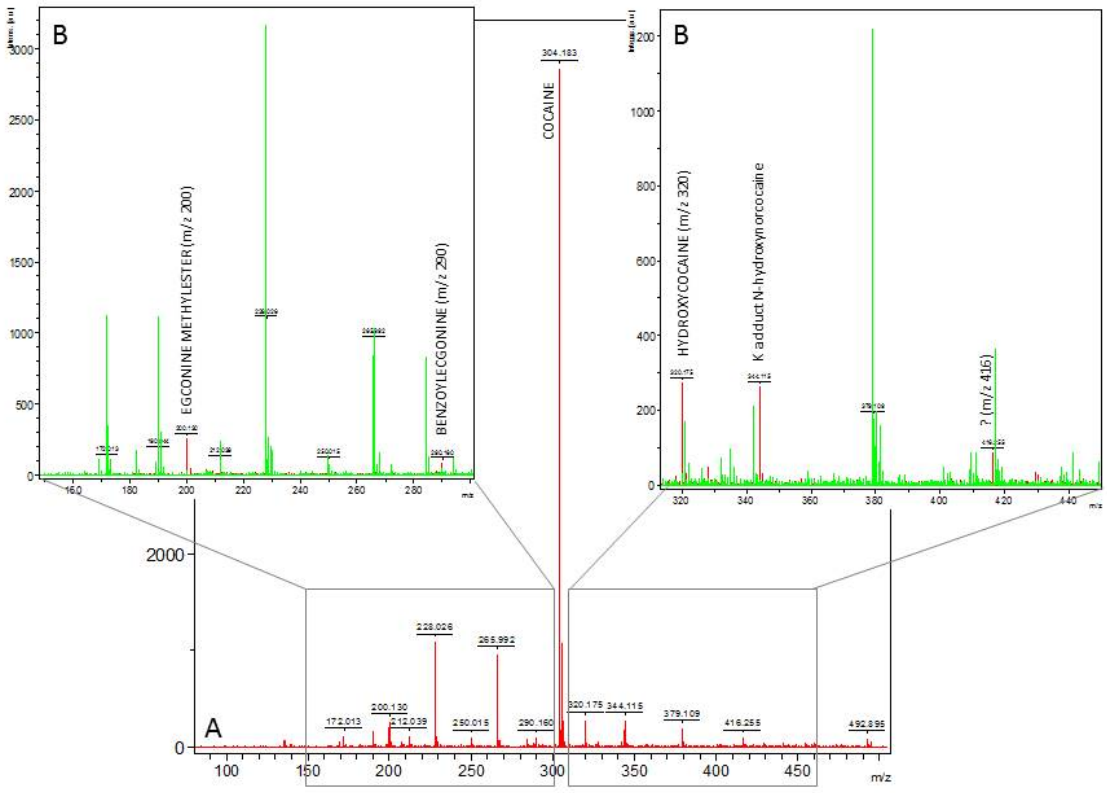

Figure 4: ***A.*** *MS spectrum of cocaine standard 1 mg/mL* ***B.*** *Green: MS spectrum cocaine standard before 24 h reaction with 30% hydrogen peroxide (1/1 (v/v)), Red: MS spectrum after 24 h reaction with 30% hydrogen peroxide.*

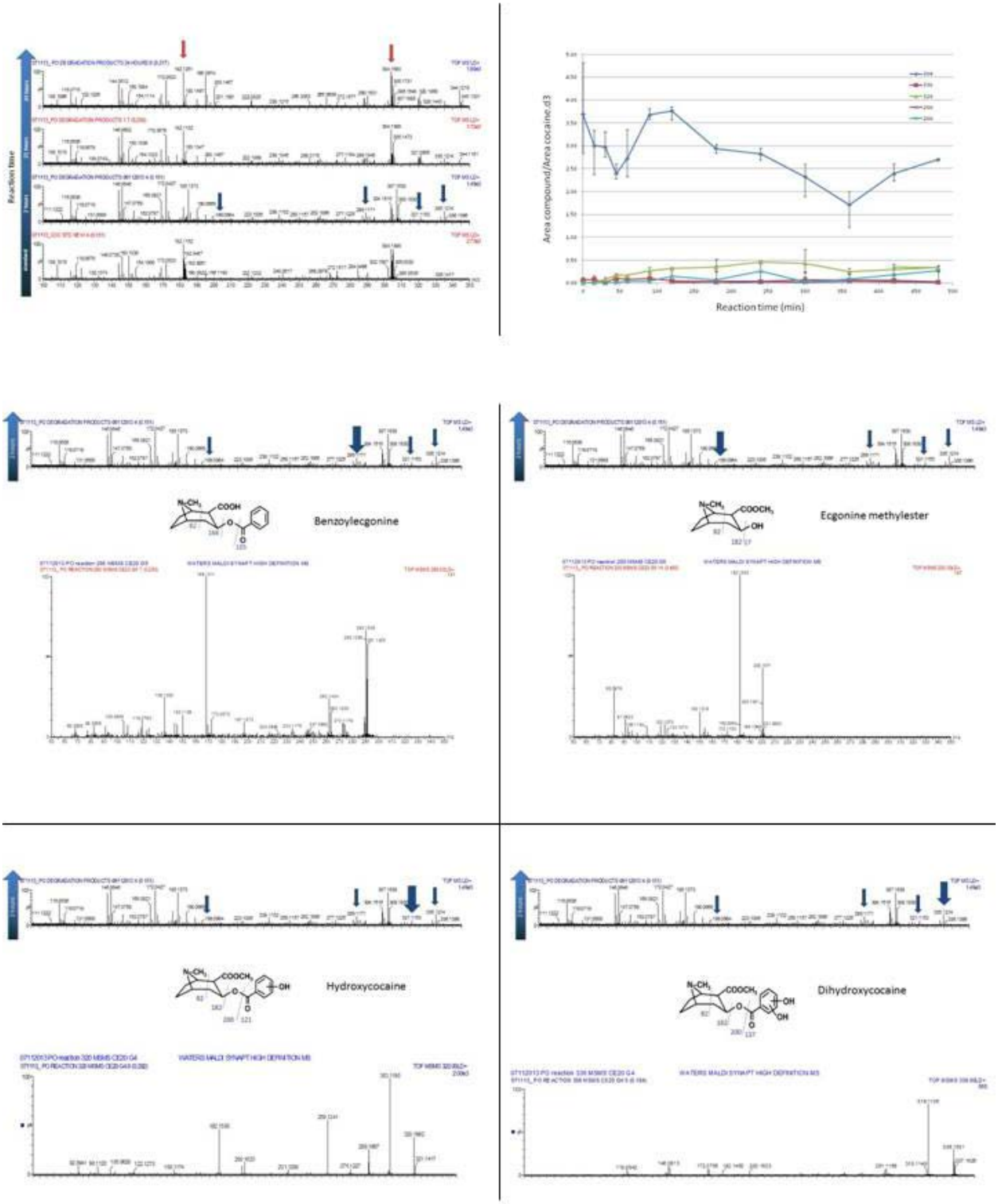

Figure 5: Top left: MS spectra of cocaine after 2, 21 and 24 hours reaction with 30% hydrogen peroxide at 37°C showing degradation products already present after 2 hours (blue arrows) and the remaining presence of cocaine after 24 hours (red arrows). The degradation of cocaine within the first 8 hours is shown by the timeline (Top right). This timeline confirms the remaining presence of cocaine after multiple hours reacting with hydrogen peroxide. Degradation products are formed in small concentrations compared to the original cocaine concentration already

indicating that the loss of detectability of cocaine can not only be attributed to the formation of reaction products. MS/MS spectra identifying four reaction products are shown below.

The described loss of capability to show the presence of cocaine in contaminated as well as users hair after peroxide treatment might be caused by the fact that cocaine is degraded into the described reaction products. Therefore, these products might be an alternative in order to prove earlier cocaine incorporation in hair. In figure 6, the described reaction products were imaged in 14 hours cocaine contaminated hairs, after bleaching for 15 minutes, using MS/MS mode. Positive (shown in figure) as well as negative mode were not able to detect the described reaction products.

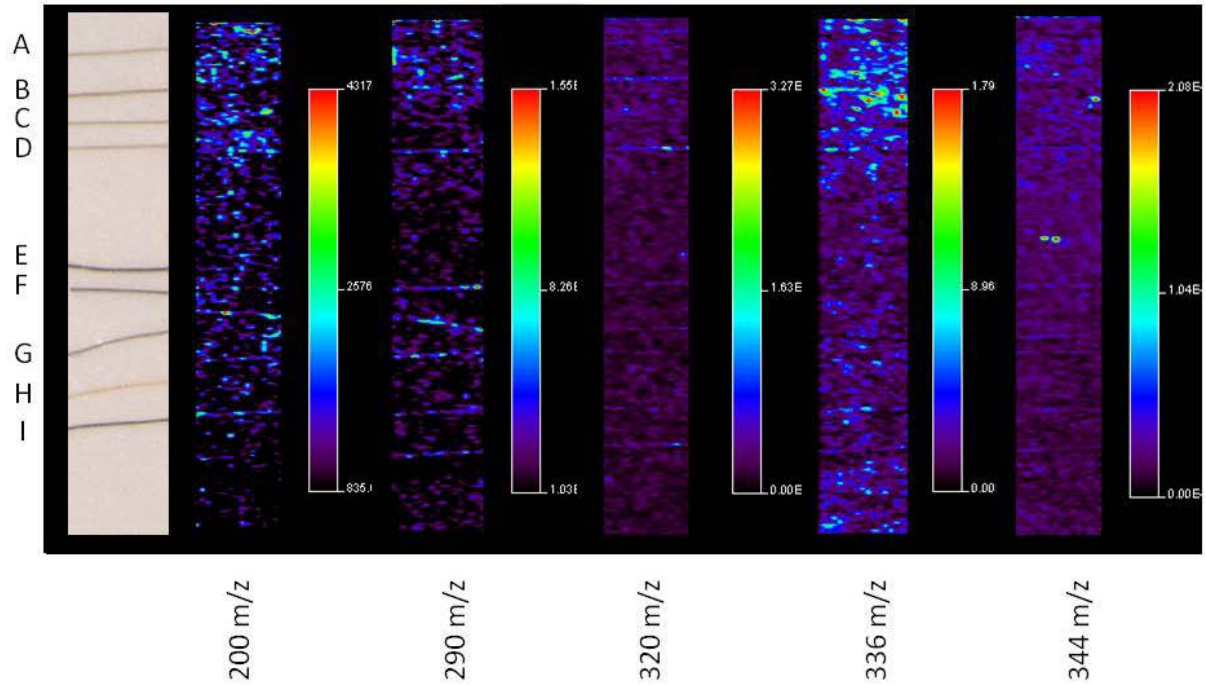

Figure 6: MS/MS images of the distribution of hydrogen peroxide reaction products in contaminated hairs. ***A.*** *Blank hair* ***B.*** *14 hours cocaine contaminated 1mg/mL* ***C.*** *14 hours 1mg/mL cocaine contaminated hair after 15 min bleaching, not washed* ***D.*** *14 hours 1 mg/mL cocaine contaminated hair after 15 min bleaching and finally 20 s washed with water* ***E.*** *cocaine user* ***F.*** *cocaine user after 15 min bleaching, not washed* ***G.*** *cocaine user after 15 min bleaching and finally 20 s washed with water* ***H.*** *cocaine user 42 hours bleached* ***I.*** *cocaine user, unbleached and 20 s washed with water*

Two possibilities might explain the absence of cocaine and its reaction products: 1) more hydrophilic reaction products are extracted from the hair by the hydrogen peroxide solution or 2) the hydrophilic reaction products are washed out after hydrogen peroxide treatment. Since no differences are seen between the washed and unwashed hairs in figure 6, the second explanation is rather unlikely since one expect to see reaction products after bleaching but before washing the hair. Nevertheless, in order to verify both hypotheses, peroxide as well as wash solution from a bleached users hair were tested for the presence of these reaction products by mixing the solution with CHCA matrix (1/1) and spotting 1 µL on the target plate (Figure 7 and 8). Surprisingly, both hydrogen peroxide as well as wash solution contain cocaine as well as some reaction products. Only trace amounts are found in the second (wash) solution. These observations cannot be explained by the limit of detection since hydrogen peroxide as well as wash solution can not contain more cocaine and reaction products than originally present in the hair. Moreover, both solutions contain a dilution of the products of interest meaning that cocaine as well as reaction products are indeed removed from the hair. Remarkably, the intensity of cocaine in hydrogen peroxide as well as wash solution is higher than the intensity of the reaction products.

These findings indicate that the described reaction of cocaine into reaction products is only partly responsible for the loss of detectable cocaine in bleached users hair. Apparently, hydrogen peroxide has two main effects on the incorporated cocaine in hair: 1) it oxidizes at least part of the incorporated cocaine, producing a number of more hydrophilic reaction products which are removed easily by washing the hair 2) hydrogen peroxide per se (partly) removes cocaine and reaction products. Both effects finally lead to a loss of detectability of cocaine in users hair.

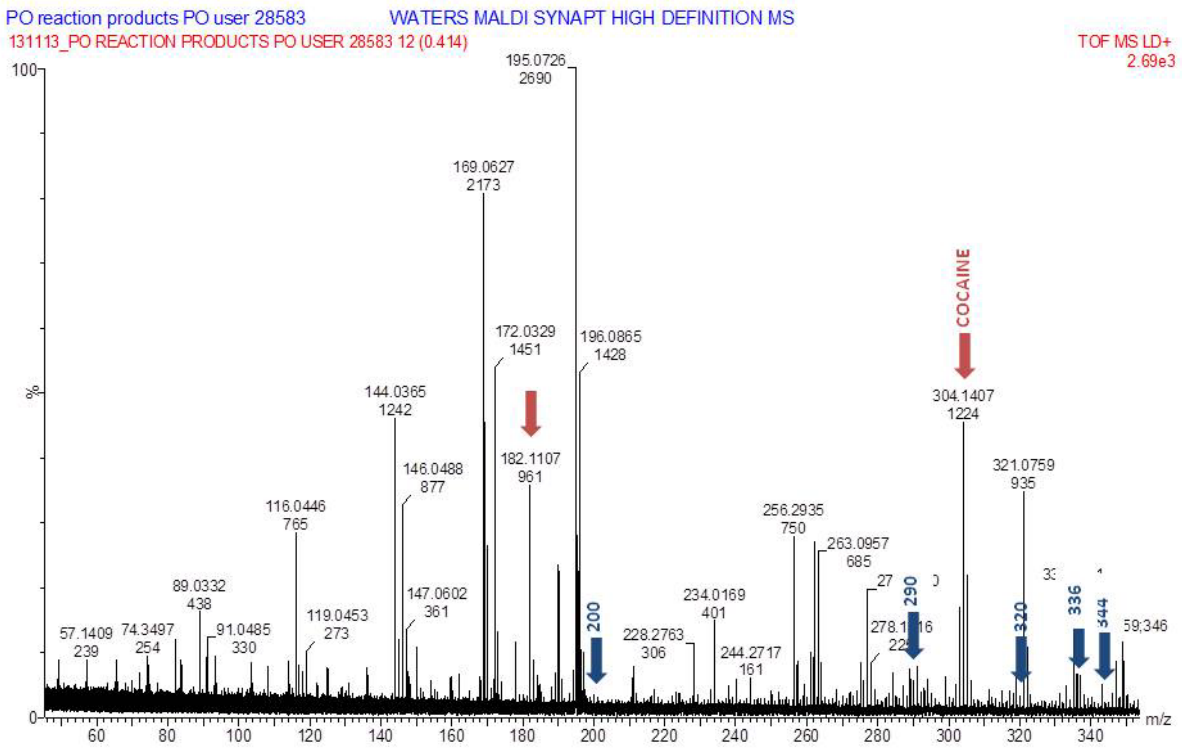

Figure 7: MS spectrum of ***peroxide solution*** *after 42 hours peroxide treatment of cocaine users hair. Arrows indicate cocaine (m/z 304) and product ion (m/z 182), ecgonine methylester (m/z 200), benzoylecgonine (m/z 290), hydroxycocaine (m/z 320), ecgonine methylester (m/z 336) and K-adduct of N-hydroxynorcocaine (m/z 344).*

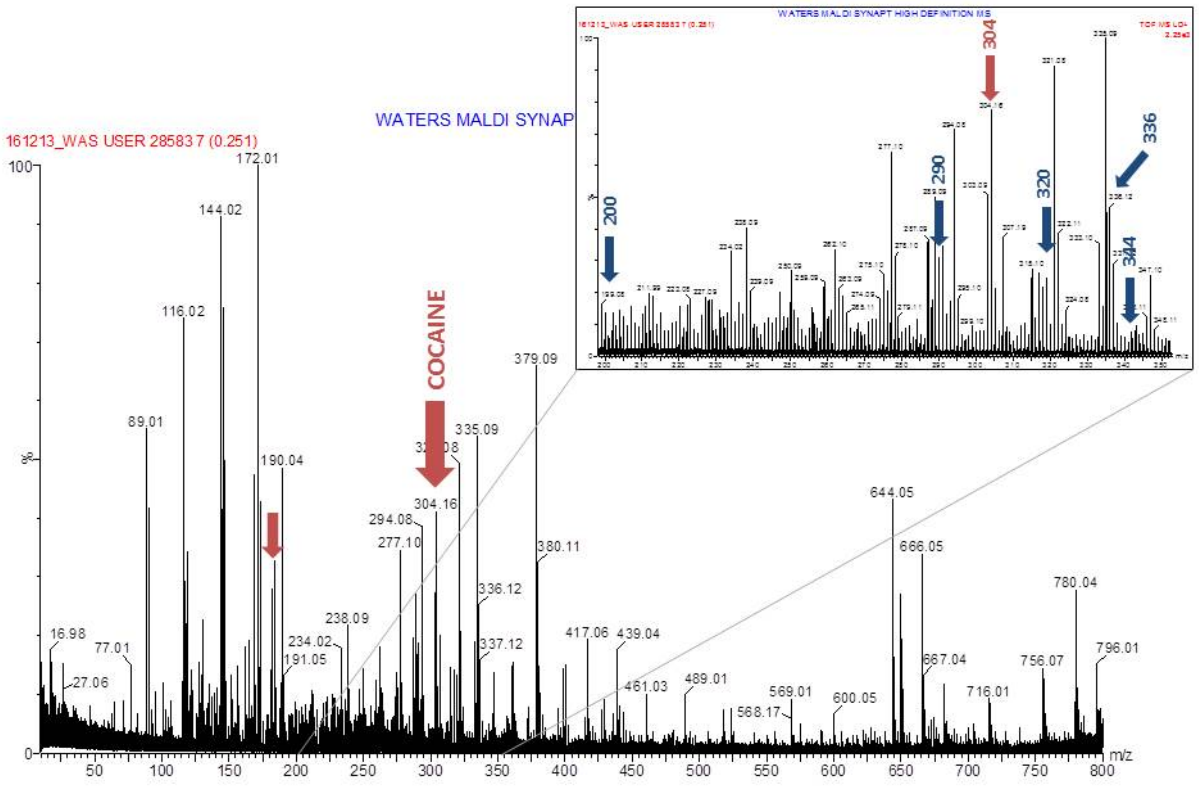

*Figure 8: MS spectrum of **wash solution** after 42 hours peroxide treatment of cocaine users hair. Arrows indicate cocaine (m/z 304) and product ion (m/z 182), ecgonine methylester (m/z 200), benzoylecgonine (m/z 290), hydroxycocaine (m/z 320) and ecgonine methylester (m/z 336).*

The question remains why cocaine itself is more easily washed out after bleaching. A possible hypothesis might be that hydrogen peroxide influences the binding of cocaine to melanin. If hydrogen peroxide breaks this binding, cocaine is more easily washed out, explaining its loss of detectability. To prove this hypothesis, a solution of cocaine and melanin was tested and compared to the MS spectrum of the same mixture after adding 30% hydrogen peroxide and incubating at room temperature for 15 minutes. As shown in figure 9, the cocaine-melanin complex peak (m/z 622.92) disappears after peroxide incubation, indicating the stated hypothesis that hydrogen peroxide most likely breaks the melanin-cocaine binding and thus making cocaine highly sensitive to removal by washing procedures.

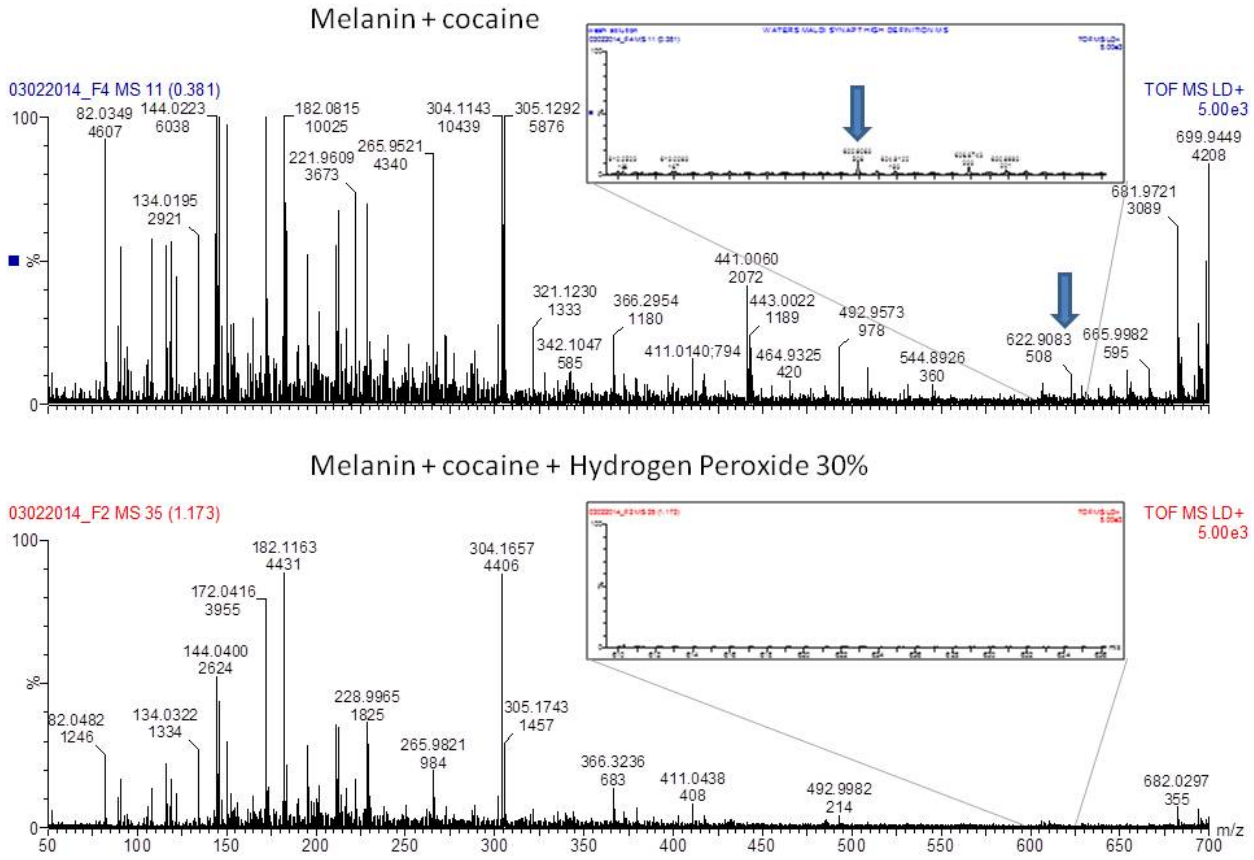

Figure 9: MS spectra of cocaine-melanin mixture (top) and cocaine-melanin-hydrogen peroxide mixture (bottom). m/z 622.92 showing the cocaine-melanin binding which disappears in the MS spectrum after adding 30% hydrogen peroxide.

CONCLUSION

In this paper, we demonstrated that mass spectrometric imaging is a useful tool in studying the effect of cosmetic treatment on cocaine concentration in (user) hair. Taking all shown results into considderation, bleaching hair with hydrogen peroxide decreases the detectability of cocaine in user hair, most likely since melanin-cocaine bonds are broken and cocaine is at least partly degraded into reaction products. Unbound cocaine as well as the more hydrophilic reaction products, including benzoylecgonine, are easily washed out, thus remove any evidence of cocaine use.

Although benzoylecgonine is often used in the discrimination between user and external contamination, the shown loss of detectable benzoylecgonine in bleached hair should be taken into account interpreting hair analysis results. Other previously proposed discriminators such as norcocaine and cocaethylene were not studied here. Nevertheless, taking into consideration that these cocaine metabolites are described to be incorporated in hair by melanin binding [22], we presume that the described melanin binding is also broken, making the polar metabolites more sensitive to washout.

These findings are of great importance in the interpretation of forensic cocaine hair analysis. Despite numerous previous studies which describe a degradation of cocaine after peroxide treatment [1, 2, 6, 23], it still remained unclear whether cocaine and/or reaction products are detectable in hair after bleaching. Here, we show evidence that peroxide treatment can, even after 15 minutes, completely remove incorporated cocaine and degradation products from the hair, making it impossible to identify cocaine users by hair analysis. The remark should be made that the cosmetic treatment on cut hair might have less potent effects when compared with treated head hair. Nevertheless, since bleaching was shown to drastically decrease the melanin content in both cut as well as head hair and thus the melanin-cocaine bond is broken, we believe the loss of detectable cocaine will be comparable. However, comparative MALDI-experiments between cosmetic treatment on cut and uncut hair might be employed to study the difference in cocaine loss.

We conclude that results obtained by hair analysis should be interpreted carefully and one should take into consideration whether hair samples were treated with any kind of oxidizing product before drawing any forensic conclusion.

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