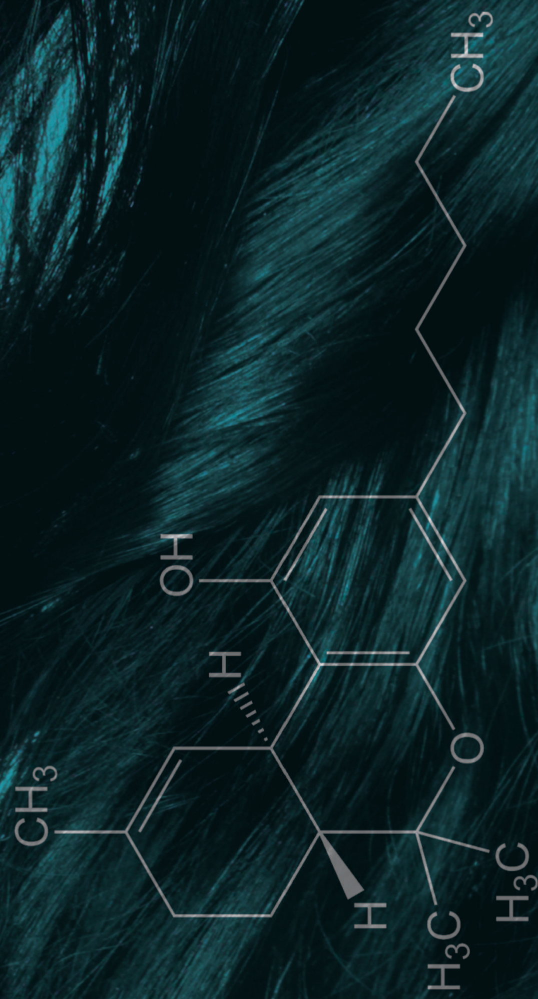
Improved Forensic Hair Evidence for Drugs of Abuse by Mass Spectrometry



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Thesis

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List of abbreviations

APCI – atmospheric pressure chemical ionization

APPI – atmospheric pressure photoionization

ASE – accelerated solvent extraction

BZE – benzoylecgonine

CBD – cannabidiol

CBN – cannabinol

CE – capillary electrophoresis

CID – collision-induced dissociation

DAPCI – desorption atmospheric pressure chemical ionization

DART – direct analysis in real time

DCBI – desorption corona beam ionization

DESI – desorption electrospray ionization

EIC – extracted ion chronogram

FWHM – full width at half maximum

GC – gas chromatography

GHB – *gamma*-hydroxybutyric acid

HCD – higher–energy collisional dissociation

HRMS – high resolution mass spectrometry

LAESI – laser ablation electrospray ionization

LC – liquid chromatography

LLE – liquid-liquid extraction

LOD – limit of detection

LTP – low-temperature plasma

MALDI – matrix-assisted laser desorption/ionization

MDMA – 3,4-methylenedioxymethamphetamine

MetA–SIMS – metal assisted-secondary ion mass spectrometry

MMS – matrix-matched standards

MRM – multiple reaction monitoring

MS – mass spectrometry

MS/MS – tandem mass spectrometry

NCE – normalized collision energy

PADI – plasma assisted desorption ionization

PMMA – *p*-methoxymethylamphetamine

RSD – relative standard deviation

SDS – sodium dodecyl sulfate

SEM – scanning electron microscopy

SFE – supercritical fluid extraction

SoHT – Society of Hair Testing

SPE – solid phase extraction

SRM – selected reaction monitoring

THCA–A – Δ^9-tetrahydrocannabinolic acid

THC – Δ^9-tetrahydrocannabinol

THC–COOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

TIC – total ion current

TLC – thin layer chromatography

TOF – time-of-flight

TWIM – travelling wave ion mobility

U(H)PLC – ultra(high)-performance liquid chromatography

Chapter 1

General Introduction

Analytical chemistry plays an important role in forensic toxicology. Blood, urine and other body fluids are very useful sources of toxicological information on both intentional and unintentional abuse of substances. However, when a longer time frame of drug use is of interest, e.g., in drug abuse cases, the detection window provided by body fluids can be insufficient. Cocaine, for example, can be detected in body fluids for only several days after use.[1] As an alternative to body fluids, hair can provide such an extended detection window, allowing retrospective monitoring of drug use for up to several months to even years before sample collection. Over the past decades, hair analysis has advanced from early usage like the detection of arsenic in Napoleon's hair[2] towards a valuable tool with applications ranging from obtaining retrospective timelines of drug administration in drug-facilitated crime evidence to doping monitoring. Other examples can be found in workplace drug testing, investigating exposure of children to drugs of abuse in child protection cases, and in veterinary control.[3-9]

Forensic hair evidence and analysis

Hair is an interesting matrix in forensics not only because of the prolonged detection window compared to body fluids, but also because sampling is non-invasive and hair samples can be easily stored.[10] Furthermore, hair can provide a chronological timeline of exposure to substances by the analysis of longitudinal hair segments.

Hair anatomy and physiology

Hair is a complex structure, consisting of several layers of different material. The hair shaft consists of overlapping cuticle cells covering the core of the hair containing the cortex and the central medulla (Figure 1A). The cuticle protects the inner regions of the hair, but can be damaged by outside sources like mechanical friction, heat or chemicals. Most of the hair is constituted by the cortex composed of long spindle-shaped, keratinized cells, which also contain pigment granules responsible for the color of hair with melanin as the most common type of pigment molecules.[4,11,12] The core of the cortex contains condensed cells, which after dehydration leave vacuoles forming the central medulla. Thick animal hair generally has a bigger medulla, while the medulla only compromises a small percentage of, thinner, human hair.[11]

Hair growth is induced from the follicle, a bulb-like skin organ surrounded by a capillary system that provides metabolic material.[13] The outer root sheath, a part of the epidermis, surrounds the inner root sheath region of the follicle. Division of cells at the bottom of the follicle bulb, just above the papilla, induces an upward movement of vertically aligned cells to the keratogenous zone where they synthesize melanin, keratinize and, after gradually dying, form a horn-like mass (Figure 1B).[11-14] Sulphur-rich keratin proteins formed during keratinization bind together through disulfide bonds and cross-linking with other proteins resulting in long fibers.[14]

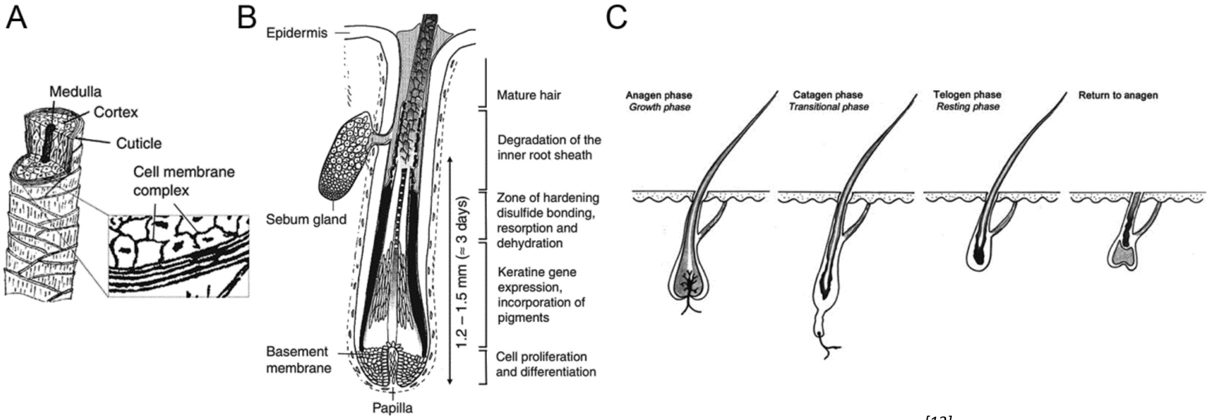


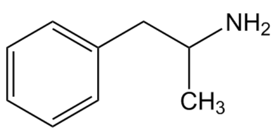
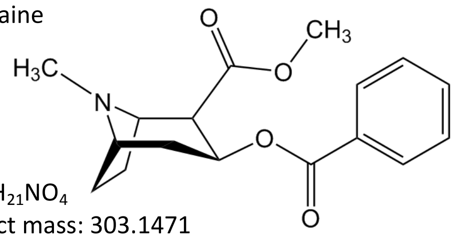
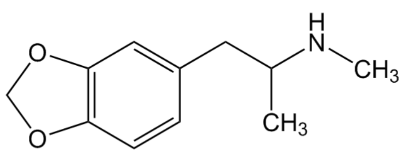
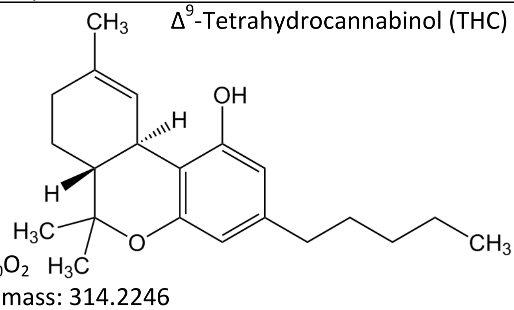
Figure 1. *A) Structure and constituents of the human hair shaft. Reprinted from [13], with permission from Elsevier. B) Formation of hair in a follicle from matrix cells on the basement membrane to the mature hair shaft. Drug incorporation from blood should occur in a 1.2 – 1.5 mm zone before completion of keratinization. Reprinted from [13], with permission from Elsevier. C) Different phases of the hair growth cycle. Reprinted from [12], with permission from John Wiley and Sons.*

The growth cycle of hair consists of three different stages: the anagen (growth), catagen (regression) and telogen (resting) phase (Figure 1C). Scalp hairs grow for 2-6 years during the anagen phase, in which rapid division of cells takes place in the follicle causing growth of the hair.[14] In the following regression phase, cell division stops causing the hair shaft to become fully keratinized and the follicle to become shorter.[11,14] During the 2-3 weeks in the catagen phase, hair starts degenerating and the club hair, a brush-like keratinized structure, is formed at the base of the hair shaft. In the following telogen phase, hair stops growing completely and only the root of the hair is anchored in the follicle. After the hair falls out, a new hair in the anagen phase starts to grow.[11,12,14] The different growth phases occur simultaneously in different hairs, i.e., not all hairs grow synchronous. 80-90% of the scalps hairs are in the anagen phase, 2% in the catagen phase and the remaining 18-20% in the telogen phase.[11,14] Although different hair growth rates have been reported, generally ranging from 0.6 to 1.5 cm/month,[7] the use of a hair growth rate of 1.0 cm/month is recommended by the Society of Hair Testing (SoHT).[15]

Drug incorporation

On a molecular level, hair consist of 65-95% protein, 15-35% water and 1-9% lipids, mainly originating from sebum and secretions of apocrine glands, and 0.25-0.95% minerals. Next to this, hair may contain compounds, such as the drugs of abuse shown in Table 1, which are incorporated into the hair matrix via different routes. Although the incorporation of compounds is not fully understood, the main incorporation model is considered to be via passive diffusion from the bloodstream into the growing hair (Figure 2A).[13] Administered drugs travel through the body after absorption into the bloodstream and reach the capillary blood supply of the follicle. Passive diffusion of the drugs into the dividing cells forming new hair matrix causes them to be incorporated into the hair shaft.[7,14] In addition to the concentration of a drug in blood, their physiochemical properties, more specific the lipophilicity and basicity, play an important role. Neutral lipophilic molecules can penetrate the cell membrane more easily, resulting in a higher incorporation rate. The pH inside the cells forming the hair matrix, the keratinocytes and melanocytes, is below the physiological pH of blood plasma. This pH gradient promotes diffusion of deprotonated basic drugs into the matrix cells. The lower pH inside the cells will cause protonation of the basic drugs and limit diffusion back into the plasma (Figure 2B). Basic drugs are therefore incorporated at higher rates.[7,13,16] Another factor influencing the incorporation rate is the ability of drugs to bind to cell proteins, particularly to melanin, causing pigmented hair to contain higher basic drug concentrations.[13]

Table 1. *Commonly used drugs of abuse.*

Amphetamine  $C_9H_{13}N$ Exact mass: 135.1048 pKa (BH^+): 9.8	Cocaine  $C_{17}H_{21}NO_4$ Exact mass: 303.1471 pKa (BH^+): 8.6
3,4-Methylenedioxymethamphetamine (MDMA)  $C_{11}H_{15}NO_2$ Exact mass: 193.1103 pKa (BH^+): 9.9	Δ^9-Tetrahydrocannabinol (THC)  $C_{21}H_{30}O_2$ Exact mass: 314.2246 pKa (AH): 10.6

Another incorporation pathway involves the diffusion of compounds into the hair shaft from sweat or sebum (Figure 2A). Many drugs of abuse have been detected in sweat after intake, often in higher concentrations than in blood. Incorporation from these body fluids can happen both before and after the hair emerged from the skin via secretions of the apocrine and sebaceous glands and sweat.[17] In addition, incorporation during hair shaft formation can take place originating from delayed release of compounds from tissue. As with incorporation from the bloodstream, these incorporation pathways are very much drug-specific.[16,17]

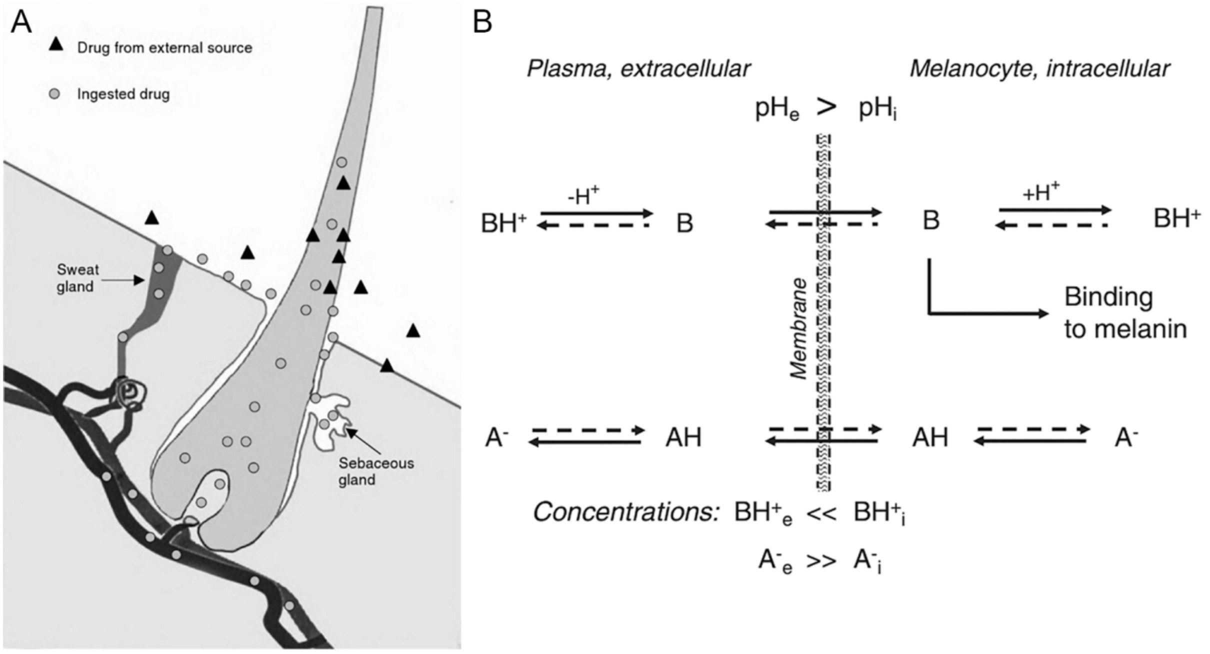


Figure 2. *A) Three pathways of drug incorporation. Ingested drugs can enter the hair from the bloodstream feeding the dermal papilla as well as by sweat and sebum bathing the mature hair fiber. External drugs from vapors or powders may also incorporate into the mature hair fiber. Reprinted from [4], with permission from Taylor & Francis Group. B) Effect of basic or acidic properties on drug incorporation rate in hair. A=acidic drug; B=basic drug; e=extra-cellular; i=intra-cellular. The lower pH in melanocytes and binding to melanin lead to an accumulation of basic drugs in pigmented hair. Reprinted from [13], with permission from Elsevier.*

External contamination

Next to incorporation from endogenous sources, compounds can also deposit onto the hair from the environment by (passive) external contamination (Figure 2A). The use of cannabis for example, rolling a cannabis cigarette and smoking thereof, induces at least two types of contamination: from smoke and via indirect contact with contaminated hands. Drugs present at the hair surface following external exposure can penetrate the hair after hair swelling under the influence of water (sweat) and/or via porous or damaged cuticles.[18] Contaminated hair samples may cause false-positive results, which can have severe consequences when hair evidence is used in court cases or workplace drug testing. Therefore, the SoHT recommends the detection of endogenously formed metabolites to prove drug use over external contamination and the use of cut-off values. These cut-off values are compound dependent and enable identification of chronic drug use.[10]

Even though most of the issues regarding external contamination can be avoided by the analysis of endogenously formed metabolites, like benzoylecgonine (BZE) for cocaine or 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) for cannabis, this is not always possible. Some metabolites are hardly formed while other metabolites are incorporated at low rates, both leading to very low metabolite concentrations in hair, in turn causing analytical challenges.[4,7]

Sampling

Proper sampling is of great importance to avoid pre-analytical errors. Collection of two pencil-thick locks of hair, one for initial analysis and one for replicate analysis or tests at a second laboratory, from the back of the head, the posterior vertex region, is recommended. Sampling time and conditions should be properly documented and, when the hair sample will be used for segmented analysis, alignment of the root end of the sample is crucial to assure the highest retrospective timeline accuracy. When applicable, the hair sample will be segmented into 1 – 3 cm segments starting from the hair root.[10] The segmented sub-samples are analyzed individually to obtain a retrospective timeline of drug exposure.

Decontamination protocols

Next to the use of cut-off values and detection of endogenously formed metabolites, an additional measure against false-positives caused by external contamination is the use of decontamination protocols to remove contamination from the hair sample, without removing endogenously incorporated compounds. Decontamination protocols described in published hair analysis studies show different strategies: washing with organic solvents, like methanol or dichloromethane, aqueous detergent or buffer solutions and protocols combining organic and aqueous washing steps. Although several more extensive studies have been published investigating decontamination strategies, it can be stated that each compound class will have a specific most effective wash protocol and no consensus has been reached so far on the most suitable decontamination protocol for each compound class. The SoHT recommends a decontamination strategy including an organic solvent wash, to remove oils and/or greasy products, and aqueous wash steps, needed to remove more deeply penetrated contamination.[10,19] The analysis of wash solvents can be used as an additional source of information regarding the ratio of external contamination and incorporated compounds.[14,20] With a proper decontamination strategy, the risk of external contamination causing false-positive results can be minimized, however, incorporated compounds can be removed by too thorough wash protocols and careful interpretation of hair analysis results is advised.[21,22]

Sample preparation and analysis methods

After decontamination, hair samples need further pretreatment (Figure 3) to extract the targeted compounds from the hair matrix. The hair sample is cut into small fragments (1-3 mm) or pulverized to generate a large surface area. Depending of the physiochemical properties of the class of compounds, they can be extracted from hair by organic solvents, aqueous solutions, or buffer solutions. Another option is to digest the hair matrix followed by extraction of the compounds of interest. Hair can be digested using different procedures: with alkaline or acidic solutions, with reducing agents or with enzymes such as pronase or a β-glucuronides/arylsulfatase mixture.[3,13,16] When necessary, clean-up and concentration steps like liquid-liquid extraction (LLE) and solid phase extraction (SPE) can be used prior to analysis.

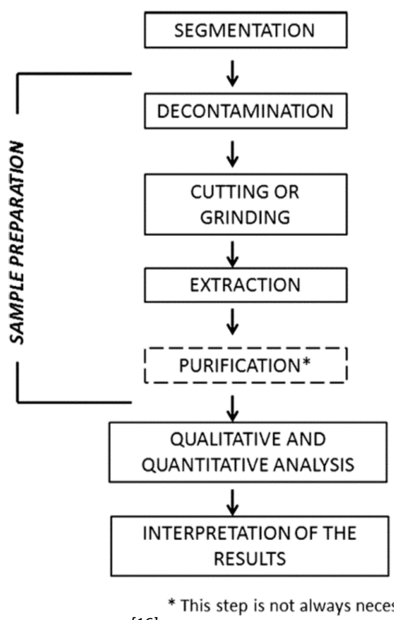


Figure 3. *Steps of hair analysis. Reprinted from [16], with permission from Elsevier.*

Conventional hair analysis involves immunoassays, liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). Immunoassays can be highly sensitive, although cross reactivity with metabolites or other compounds should be taken into account. In most cases, immunoassays are only used as preliminary test, followed by a second analysis using a different technique.[14] Chromatographic techniques are very powerful screening methods, by their ability to cover a wide range of analytes in one run.[7] Many hair analysis methods using GC have been published over the last decades, however, LC is more and more being used. Especially for polar compounds, LC is very suitable without the need of a derivatization step as with GC. In addition, CE has also been successfully applied for the determination of drugs and metabolites in hair. Mass spectrometry (MS) has been widely accepted as the detection method of choice,[5] although other detectors like diode array detection are still used for screening and quantification.[8]

Recent advances in hair analysis

Over the past few years, several analytical advances have been made in the field of hair analysis, starting with the application of matrix-assisted laser desorption/ionization (MALDI)-MS as a fast screening procedure for cocaine and its metabolites in hair extracts.[23,24] First reported in 2011,[25-27] MALDI-MS imaging has been used to analyze single hairs (Figure 4) and longitudinal cross-sections thereof to obtain very detailed MS images of the drug distribution throughout a hair utilizing the excellent spatial resolution of MALDI (30 µm). Reported imaging methods mostly involve targeted analysis of cocaine and metabolites, but detection of other drugs like methamphetamine, cannabinoids, ketamine, zolpidem and tilidine has also been reported.[25-37] Next to MALDI, the application of laser ablation electrospray ionization (LAESI)[38] and metal assisted-secondary ion MS (MetA-SIMS)[33,37] to single hair analysis with high spatial resolution has been demonstrated.

Retrospective assessment of the time of drug administration by single hair analysis, however, is limited since individual hairs do not grow simultaneously, i.e., each hair can be in a different growth phase. Musshoff *et al*.[28] report the analysis of four single hairs from one individual: two hairs were found to be positive for cocaine, its metabolites and cannabinoids, while the other two hairs yielded negative analysis results. The imaging of multiple hairs may lead to a reliable average, but this is a very time consuming process compared to conventional hair analysis methods. Nevertheless, the high spatial resolution of the MS imaging techniques can lead to very valuable insights into drug incorporation mechanisms, as reported by Kamata *et al*.[34] The incorporation of methoxyphenamine, a non-regulated analogue of methamphetamine, into the hair matrix was studied using MALDI-MS imaging and showed that incorporation of methoxyphenamine into the hair matrix originated from two entry points: the hair bulbs and the upper dermis area. The two incorporation sites resulted in two 2−3 mm long bands close together, which then grew with the hair along the hair shaft towards the distal end. This resulted in an experimental chronological resolution following single dose drug intake of about 11 days.[34]

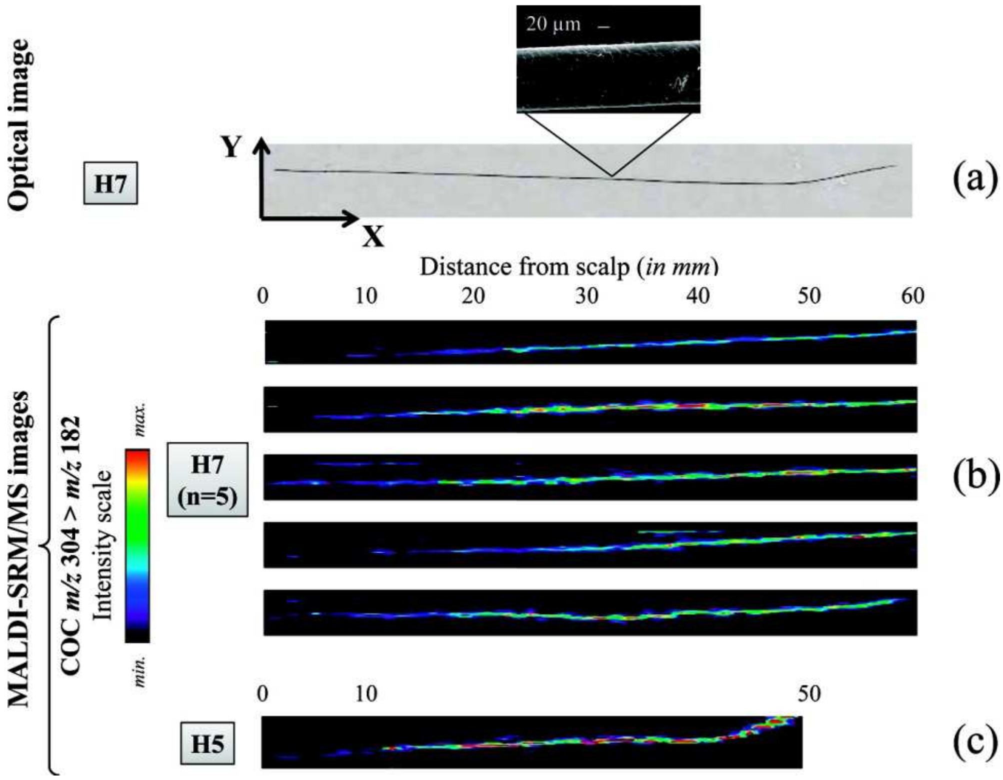


Figure 4. *Imaging of cocaine along hair samples. (a) Optical image of hair sample. MALDI-selected reaction monitoring (SRM)/MS image based on the SRM trace of cocaine (m/z 304 > m/z 182) for (b) five replicates of single hair samples from one individual and (c) single hair analysis from a second individual. Reprinted with permission from [27]. Copyright 2011 American Chemical Society.*

Applications of hair analysis

Hair analysis has applications throughout several fields. Most common are the forensic toxicological applications, where hair can be used to evaluate past (pharmaceutical) drug and alcohol use of individuals, both alive and in post-mortem cases. In the latter, the stability of drugs in hair is an additional advantage over the analysis of body fluids.[4] Most hair analysis methods focus on one or more classes of common drugs of abuse, namely, amphetamines, cocaine, opiates, cannabinoids and hallucinogens.[39] Hair analysis methods are also used to monitor alcohol abuse by the detection of ethanol metabolites, like ethyl glucuronide and fatty acid ethyl esters. Recently, the recent rise of new psychoactive substances, like synthetic cannabinoids and cathinones, caused the need to develop hair analysis methods to address this rapidly growing group of substances.[7]

Analysis of longitudinal hair segments, taking into account the hair growth rate, can be used to obtain a retrospective timeline of an individual's contact with substances. A very specific use of segmented hair analysis is in drug-facilitated crime evidence, where the time of unintentional drug administration of rape drugs, like GHB and benzodiazepines, is crucial. Next to forensic toxicological applications, hair analysis is used in workplace drug testing and doping control to investigate an individual's substance use. Where workplace drug testing is mostly focused on common drugs of abuse and anesthetics, hair analysis for doping control is targeted to substances which are prohibited out of competition, like anabolic steroids.[4,7]

The use of hair analysis is not limited to human samples, and is also used in veterinary control. An example is veterinary drug residue analysis for steroids, β-agonists and other regulated substances,[40] or for doping control in horse racing.[41] Furthermore, it can be used to assess animal well-being by the analysis of cortisol, a stress related hormone.[42,43]

Challenges in hair analysis

In addition to different incorporation mechanisms, external contamination and the limitations of single hair analysis, hair analysis comes with several, both biological and analytical, challenges. Biological, cultural and genetic reasons can cause differences in the amount of drug that is incorporated into the hair of different individuals after administration of identical amounts of that drug. Next to the effect of hair color on drug binding, several studies show that differences in morphology between African hair and Caucasian and Asian hair can cause higher incorporation rates in African hair due to higher hair shaft breakage and other structural damage.[4,44] Careful consideration of the segment length used in segmented hair analyses is also of great importance. The analysis result of each segment only provides an estimation of drug exposure during the corresponding period of hair growth.[7] With the currently used 1-3 cm segments, each data point thus represents 1-3 months of drug exposure history.[10] Another biological source of pre-analytical bias is the difference between head hair samples from the same individual. Variations up to 62% were found, indicating that even intra-individual sub-samples can yield different hair analysis results.[45] Cosmetic hair treatments, such as bleaching, can also negatively influence the detectability of targeted compounds.[32]

Analytical challenges arise mainly during extraction of the targeted compounds from the hair matrix. Proper extraction, following the approaches described in paragraph 1.1.6, and validation thereof is crucial to obtain reliable analysis results. The extraction procedure should therefore be extensively optimized on authentic hair samples.[6,21] The available sample amount in hair testing can be quite limited (<100 mg), especially when a portion of the sample has to be stored for confirmatory testing,[6] causing sensitivity problems when compounds are incorporated in very low amounts. In addition, the growing list of new psychoactive substances asks for continuous expansion of the analysis methods. Consequently, multi-target analysis and screening procedures are urgently needed.[6,21]

Ambient mass spectrometry

Over the last decade, several MS ionization techniques enabling direct ionization at atmospheric pressure to analyze samples in their native state have been introduced. With their publication about desorption electrospray ionization (DESI), Takáts *et al*.[46] described the first ambient ionization technique in 2004. The ability of directly analyzing the surface of a wide range of objects gave rise to many applications and was the start of the rapid development of the field of ambient mass spectrometry. The main advantage of ambient mass spectrometry is the limited sample preparation or complete lack thereof, enabling very short analysis times and high throughput. Matrix effects are low and samples with high salt content can be analyzed as such. In addition, most methods use soft ionization mechanisms, causing little to no molecular fragmentation during ionization.[47,48] However, ambient ionization also comes with limitations. Most ambient ionization methods have a limited mass range and, where conventional analysis techniques use sample clean-up and/or chromatographic separation to reduce the error caused by matrix effects, direct analysis of samples using ambient ionization generally yields poor relative standard deviations (RSD). This is mainly caused by the sample complexity as everything present on the sample can be ionized. The ambient conditions can be an additional source of error: changes in temperature and humidity, and the presence of possible contaminants in the air will negatively influence the reproducibility and precision.[49] Internal standards can be used to overcome these challenges, however, application of internal standards to a sample surface to be analyzed by ambient ionization is challenging, and quantification possibilities are thus limited.

After the introduction of DESI, many ambient ionization techniques have been developed, which have been classified into seven categories by Monge *et al*.[50] based on their desorption/ionization mechanism, although other subdivisions have also been proposed[51-53]: 1) solid-liquid extraction based, 2) plasma-based, 3) two-step thermal or mechanical desorption/ablation, 4) two-step laser based desorption/ablation, 5) acoustic desorption, 6) multimode techniques, and 7) one-of-a-kind techniques that do not fit the previous categories. The majority of the published techniques fall within the first four categories.[50] All techniques have specific applications and their advantages and disadvantages. Laser based techniques can be used with outstanding spatial resolutions thanks to the small laser spot size, however, they are less suitable for instant analysis of larger surface areas. A solid-liquid extraction based technique like DESI is applicable to very polar compounds and has a high mass range, but the targeted surface needs some degree of wettability and electro-conductivity. Most interesting for the ambient analysis of hair samples are therefore plasma-based ionization techniques, of which direct analysis in real time (DART)[54] is considered as the most established and commercially available technique with a wide range of applications. Plasma-based ionization techniques either use plasma-sample interactions or involve interaction of gaseous reagent species, formed by a plasma source, with the sample.[50,53] Examples of techniques using direct plasma-sample interactions are desorption corona beam ionization (DCBI)[55] and low-temperature plasma (LTP).[56] The stability of the plasma can, unfortunately, cause difficulties when it is directly used to analyze a sample.[57] By use of plasma-formed gaseous reagent species, more stable and controllable ionization conditions can be obtained. In addition, gas-based techniques might be able to not only desorb analytes from the surface of the sample, but also penetrate a sample to desorb analytes from below the surface. Next to DART, desorption atmospheric pressure chemical ionization (DAPCI)[58] is another example of an ambient ionization technique using gaseous reagent species. Nevertheless, DART has, next to its commercial availability with many configurations, the unique feature that ionic species are removed from the gas stream before interaction with the surface, thus reducing the risk of loss of ionized analyte species through ion-ion recombination.[57]

DART instrumentation

DART ionization was first described by Cody *et al*. in 2005.[54] They demonstrated its use to rapidly analyze a variety of compounds in liquids and on surfaces by ionization using metastable species of a heated gas.[54] Helium is primarily used for DART, although the use of nitrogen and argon has also been reported.[59-61] Inside the DART ionization source, the gas flows through several chambers (Figure 5). A corona discharge between a needle electrode and a perforated disk electrode in the first chamber produces ions, electrons and excited atoms of the selected gas. The second and third chambers are used to, respectively, remove ions using a perforated electrode and, optionally, heat the gas to temperatures from 50 to 550 °C. The DART source can be used in two modes: by pointing the gas flow directly towards the mass spectrometer inlet (transmission mode), allowing analysis of a sample by placing it between the DART source and the mass spectrometer, and by placing the DART source at an angle and reflection of the gas flow from a surface to the mass spectrometer.[47,54]

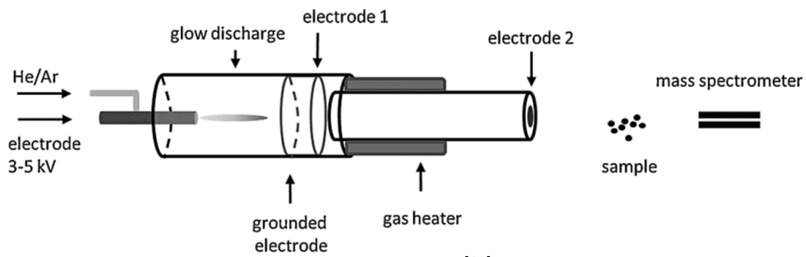


Figure 5. *Schematic diagram of DART source. Reprinted from [62], with permission from John Wiley and Sons.*

DART ionization mechanism

Helium is the most efficient ionization gas in DART ionization since excited neutral helium species store 19.8 eV of energy, which is higher than the ionization energy of atmospheric water and most relevant molecules.[47] The use of other ionization gasses has also been reported: use of nitrogen reduces costs, while argon as ionization gas can result in better selectivity and lower in-source fragmentation. Ionization efficiency, however, is lower for most compounds compared to the use of helium since the energy of the excited states of nitrogen and argon is not high enough to ionize atmospheric water.[54,59-62]

Prior to ionization, analyte ions need to be present in the gas phase. This can be achieved by evaporation or thermal desorption facilitated by the heated gas provided by the DART source.[61] The subsequent gas-phase ionization can generate positive and negative ions following several parallel pathways closely related to those in atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).[47] For positive ionization, metastable helium species, He^*, exiting the DART source can interact with other gaseous species, including analyte molecules (M). This process, called Penning ionization, yields molecular ions ($M^{+\bullet}$) (1) when the ionization energy of the analyte is lower than the internal energy of the excited helium (19.8 eV).[54] Molecular ions can, in addition to direct formation via Penning ionization, be formed by charge transfer from $N_4^{+\bullet}$, $O_2^{+\bullet}$ and NO^+ formed from ambient gases (2).[47]

$$\text{(1)}\quad He(2^3S)^* + M \rightarrow He(1^1S) + M^{+\bullet} + e^-$$

$$\text{(2)}\quad N_4^{+\bullet} + M \rightarrow 2N_2 + M^{+\bullet}$$

$$O_2^{+\bullet} + M \rightarrow O_2 + M^{+\bullet}$$

$$NO^+ + M \rightarrow NO + M^{+\bullet}$$

The most abundant ions in positive ionization DART, however, are quasi-molecular ions ($[M+H]^+$). Atmospheric water reacts with the metastable helium species, yielding Penning ionization of atmospheric water (3). The amount of gas phase collisions at atmospheric pressure is high, 10^9 to 10^{10} collisions per second, causing fast ion-molecule reactions and in the case of DART, ionized water species react with atmospheric moisture to form protonated water clusters (4).[47] These water cluster ions can yield protonated analyte molecules via proton transfer when the proton affinity of the analyte is higher than that of the ionized water clusters (5).[54,63,64]

$$\text{(3)}\quad He(2^3S)^* + H_2O \rightarrow He(1^1S) + H_2O^{+\bullet} + e^-$$

$$\text{(4)}\quad H_2O^{+\bullet} + nH_2O \rightarrow [(H_2O)_n+H]^+ + OH^\bullet$$

$$\text{(5)}\quad [(H_2O)_n+H]^+ + M \rightarrow (H_2O)_n + [M+H]^+$$

An alternative pathway of the formation of quasi-molecular ions is via a multistep mechanism involving Penning ionization of nitrogen (6). Dimers of nitrogen are formed in a so-called third body reaction (7). Hereby, a third body, N_2, is used to stabilize the product ion ($N_4^{+\bullet}$) originating from the reaction of two species, in this case $N_2^{+\bullet}$ and N_2. The radical cation, $N_4^{+\bullet}$, then ionizes atmospheric water via charge transfer (8). Again, water cluster ions are formed (4) which can lead to analyte ion formation via proton transfer as described above (5).[47,65]

$$\text{(6)}\quad He(2^3S)^* + N_2 \rightarrow He(1^1S) + N_2^{+\bullet} + e^-$$

$$\text{(7)}\quad N_2^{+\bullet} + N_2 + N_{2\,\mathit{(3rd\ body)}} \rightarrow N_4^{+\bullet} + N_{2\,\mathit{(3rd\ body)}}$$

$$\text{(8)}\quad N_4^{+\bullet} + H_2O \rightarrow 2N_2 + H_2O^{+\bullet}$$

Since DART is an ambient ionization technique, attachment of ammonia from the ambient air can be observed for some compounds. These, mostly more polar, compounds have less proton affinity than ammonia and are therefore unable to deprotonate NH_4^+, while their functional O-groups can form hydrogen bonds with NH_4^+ yielding $[M+NH_4]^+$ ions.[66]

Negative ions in DART ionization are formed via multiple mechanisms similar to the ionization mechanisms of APPI. Electrons can be formed from Penning ionization of atmospheric nitrogen (6) or neutrals (N) in the gas phase (9), or by interaction of metastable helium with a surface like the exit electrode of the DART source (10). Electron capture by atmospheric oxygen then yields $O_2^{-\bullet}$ reagent ions (11). When analytes have a stronger gas-phase acidity than HO_2, deprotonated ions ($[M-H]^-$) can be observed (12).[47,66,67]

(9) $He(2^3S)^* + N_{gas} \rightarrow He(1^1S) + N_{gas}^{+\bullet} + e^-$

(10) $He(2^3S)^* + N_{surface} \rightarrow He(1^1S) + N_{surface}^{+\bullet} + e^-$

(11) $O_2 + e^- \rightarrow O_2^{-\bullet}$

(12) $O_2^{-\bullet} + M \rightarrow [M-H]^- + HO_2^\bullet$

The $O_2^{-\bullet}$ reagent ions can also form adducts with analytes and be detected as such (13) or after dissociation into radical anions (14). In addition, other mechanisms can take place depending on the analyte properties: direct electron capture (15), dissociative electron capture (16), deprotonation by dissociation (17), and anion attachment (18). [47,67]

(13) $O_2^{-\bullet} + M \rightarrow [M+O_2]^{-\bullet}$

(14) $[M+O_2]^{-\bullet} \rightarrow M^{-\bullet} + O_2$

(15) $M + e^- \rightarrow M^{-\bullet}$

(16) $MX + e^- \rightarrow M^- + X^\bullet$

(17) $MH \rightarrow [M-H]^- + H^+$

(18) $M + X^- \rightarrow [M+X]^-$

DART-MS yields relatively simple spectra: mostly single ion species as described above, no multiply charged ions, little chance of fragmentation and no alkali-metal cation adducts.[66] Other forms of adduct formation are possible, either as side ionization mechanism or forced by the introduction of dopants into the ionization region to improve the sensitivity. Often used dopants are NH_3, CH_2Cl_2 and TFA yielding, respectively, $[M+NH_4]^+$, $[M+Cl]^-$ and $[M+CF_3COO]^-$.[47]

Applications of DART

Over the last decade, DART has become an established ionization technique with many applications. DART is most successful for, but not limited to, the analysis of molecules with a low to medium polarity and molecular weight.[68] The general *m/z* range covered by DART starts at around *m/z* 50 and goes up to *m/z* 1,200.[51] The analysis of high molecular weight molecules, even above *m/z* 5,000, was demonstrated using ionic liquids.[69] The limiting factor in DART ionization is the ability of an analyte to (thermally) desorb from the sample, without thermal degradation. Although DART is mostly used for qualitative analysis, (semi-)quantitative analysis is also possible. Liquid samples are more suited for quantification compared to solid samples, due to the possibility to add an internal standard to liquid samples.[70]

Up to 2015, over 300 papers have been published describing the use of DART ionization. Applications vary from the analysis of solid samples to liquids and gasses, however, the direct analysis of analytes from a surface without sample preparation is what makes DART so valuable. Many applications focus on the detection of drugs of abuse or counterfeit pharmaceuticals,[62] but DART has a very broad application range.[53] For example, the use of DART to assess food quality and safety has been extensively investigated.[71] Some examples hereof are analysis of pesticides from fruit and vegetable surfaces,[72] detection of melamine in powdered milk[59] and analysis of silicones released from household items.[73] Recently, the application of DART to the field of metabolomics has been explored.[49,74] Another use of DART is the analysis of, covalently bound, organic monolayers on different substrates.[75,76] The application of DART in the field of forensics is not just limited to the detection of (pharmaceutical) drugs from solid samples,[77] plant material,[78] and cotton-swab wipes,[79] DART has also be used to analyze urine samples,[80] dried blood spots,[81] and explosives.[82]

Aim of this research

The need for extensive and laborious sample preparation protocols makes conventional hair analysis a time consuming process. In addition, retrospective time of drug exposure can only be determined using quite large time frames in the order of months. New hair analysis methods involving MS imaging of single hairs show promising results, but routine use of single hair analysis is limited by differences in growth cycles between individual hairs. In this thesis, an entirely novel approach in hair analysis is investigated by the use of excited gas molecules to scan intact hair samples. The use of a state-of-the-art ambient mass spectrometry technique, DART, could directly produce a continuous retrospective timeline of drug exposure without extensive sample preparation, resulting in much shorter analysis times. Simultaneous detection of a wide range of drugs of abuse from drug user hair samples could provide an overview of drug use, while detection of metabolites could indisputably prove drug use over external contamination. Crucial to the development of any new method is a critical comparison with conventional methods, in this case to assure its usefulness in forensic applications. Since ambient MS analysis of hair will produce many more ions next to the compounds of interest, different mass analyzers should be evaluated in terms of selectivity.

External contamination is a major issue in forensic hair testing since it can cause false-positive results. Decontamination strategies are compound dependent and in current literature no consensus has been reached on which decontamination protocol should be used to remove external contamination without removing incorporated compounds.[8] Some more extensive decontamination studies have been published, most of them focusing on the removal of cocaine contamination.[83-90] Not much attention has been given to cannabis contamination, even though the use of cannabis causes a high risk of contamination via smoke or hand-to-hair contact. An extensive, evidence-based, evaluation of decontamination protocols following the SoHT guidelines for the removal of cannabis contamination would enhance the value of forensic hair evidence and provide more confidence in forensic evidence.

Next to forensic toxicology, hair analysis is a valuable tool in veterinary control of the use of banned or regulated substances and drug residues. Current methods, however, use hair obtained by shaving to determine whether or not an animal has been in contact with banned or regulated substances. This approach does not provide any insight in the time of administration, while this could provide valuable information since livestock is regularly traded. The forensic hair collection protocol of the SoHT and subsequent segmented hair analyses may be used to investigate the feasibility of obtaining timeline information for veterinary hair samples.

Outline of this thesis

Through the research described in this thesis, I strived to further develop hair analysis from a forensic, analytical and veterinary point of view. The content of each chapter is summarized below.

Chapter 2 describes the evidence-based evaluation of decontamination protocols for the removal of cannabinoid contamination. For this purpose, hair samples were contaminated with cannabinoids using different approaches. A wide range of organic solvents and aqueous solutions were tested for their decontamination properties. Combined protocols using the best performing solvents were extensively evaluated on drug user hair samples as such and after additional contamination.

In **Chapter 3**, the development of a new hair analysis method is described. DART high resolution mass spectrometry (DART-HRMS) was used to scan intact locks of hair without any sample preparation. Proof-of-principle was achieved through the detection of Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound in cannabis, in cannabis user hair samples.

A much more comprehensive DART hair scan method was developed in **Chapter 4**. Simultaneously with structural confirmation of four common drugs of abuse by data-dependent product ion scans, full scan high resolution data was recorded and used to retrospectively detect several other, less common, drugs of abuse and occasionally abused pharmaceutical drugs. Different temporal profiles of multiple compounds could be obtained by DART hair scanning of drug user hair samples.

A critical comparison of the selectivity and sensitivity of different mass analyzers for application to DART hair scanning is described in **Chapter 5**. Both spiked and drug user hair samples were analyzed using low and high resolution mass analyzers. Additionally, the use of ion mobility to enhance selectivity was investigated.

A study to investigate the feasibility of segmented hair analysis for veterinary drug residue analysis is presented in **Chapter 6**. Locks of tail hair were obtained from calves using the forensic hair sampling protocol prior to and after clenbuterol administration. Segmented hair analysis of the locks of hair generated clenbuterol profiles along the hair, which were in good correlation with the administration timeframe.

Chapter 7 contains a general discussion of the topics described in this thesis and gives an insight in the possibilities for further development of hair analysis, and more specific the developed DART hair scan method.

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Chapter 2

Evidence Based Decontamination Protocols for the Removal of External Δ^9-Tetrahydrocannabinol from Contaminated Hair

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Abstract

External contamination can cause false positive results in forensic hair testing for drugs of abuse and is therefore a major concern when hair evidence is used in court. Current literature about decontamination strategies is mainly focused on external cocaine contamination and no consensus on the best decontamination procedure for hair samples containing cannabinoids has been reached so far. In this study, different protocols with solvents, both organic as well as aqueous, were tested on blank and drug user hair for their performance on removing external cannabis contamination originating from either smoke or indirect contact with cannabis plant material. Smoke contamination was mimicked by exposing hair samples to smoke from a cannabis cigarette and indirect contact contamination by handling hair with cannabis contaminated gloves or hands. Δ^9-tetrahydrocannabinol (THC) levels in the hair samples and wash solvents were determined using liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis. Aqueous surfactant solutions removed more THC contamination compared to water, but much less than organic solvents. Methanol, dichloromethane and chloroform were most efficient in removing THC contamination. Due to its lower environmental impact, methanol was chosen as the preferred decontamination solvent. After testing of different sequential wash steps on externally contaminated blank hair, three protocols performed equally well, removing all normal level and more than 99% of unrealistically high levels of external cannabis contamination. Thorough testing on cannabis users' hair, both as such and after deliberate contamination, showed that using these protocols all contamination could be washed from the hair while no incorporated THC was removed from truly positive samples. The present study provides detailed scientific evidence in support of the recommendations of the Society of Hair Testing: a protocol using a single methanol wash followed by a single aqueous SDS solution wash, followed by a Milli-Q water rinsing step, is suggested as the preferred decontamination protocol to remove external cannabis contamination from hair while preserving the incorporated compounds.

Highlights

- External contamination mimicked at different levels.
- Wide range of solvents tested to remove external cannabis contamination.
- Decontamination protocol using a methanol wash followed by an SDS wash is recommended.
- Incorporated cannabinoids are not extracted by proposed protocols.

Introduction

Hair is an interesting matrix for forensic investigations. Sampling is non-invasive, storage is easy, hair is difficult to falsify and segmented hair analysis can provide retrospective information of time of drug intake.[1,2] External contamination, however, is a major problem in forensic hair testing. When drugs of abuse are used, other individuals than the users may come in contact with the substance via smoke or (in)direct physical contact. Especially smoked or snorted drugs of abuse like cocaine, heroin and cannabis are likely to cause external contamination[3] and could as a result yield false positive hair analysis results. Several studies have been published describing contamination of hair by drugs of abuse. While many of those studies focus on cocaine[4-11] as a result of the severe consequences of its use, cannabis contamination is also of interest.[12-19] Cannabis is extensively used in The Netherlands due to its wide and legal availability and can easily result in external contamination of non-drug users' hair, either due to passive contact with cannabis smoke or physical contact with users who handled cannabis material while preparing a joint. For example, children can be exposed to sources of cannabis when it is used by their parents, caretakers or other relatives.[16]

When hair analysis is used for drug use determination, in general two cannabinoids are monitored: Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound of cannabis, and its main metabolite 11-*nor*-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH).[20] Recently, the Auwärter group published several papers describing different sources of (synthetic) cannabinoid contamination.[14-19] Although synthetic cannabinoids differ in chemical structure from THC, contamination studies related to synthetic cannabinoids were nevertheless considered relevant. In vivo side stream smoke contamination of hair of volunteers resulted in THC concentrations up to 1.7 ng/mg,[15] which is somewhat lower than the concentrations after smoke contamination of cut hair found by Thorspecken *et al*. in their decontamination study (2.5–20 ng/mg).[13] A different study from Hutter *et al*. with side stream smoke from a synthetic cannabinoid containing cigarette resulted in contamination levels up to 0.95 ng/mg.[14] Furthermore, handling of herbal mixtures containing synthetic cannabinoids, regardless of wearing gloves, caused hair contamination levels in the sub ng/mg range.[17] An experiment in which volunteers handled cannabis plant material and did not wash their hands for at least 3 h showed that hand-to-hair contact can indeed contribute a great deal to the total amount of cannabinoids found on hair.[19] Since drug users' hair contains THC in concentrations ranging from 0.1 to 7.6 ng/mg[12,21-23] and the Society of Hair Testing (SoHT) cut-off value is 0.05 ng/mg,[20] the contamination levels observed underline that external contamination can be a serious problem in forensic hair testing.

The risk of false-positive findings can be lowered by analysis of an endogenously formed metabolite and the use of cut-off values for chronic drug use as recommended by the SoHT.[20] The main metabolite of THC, THC-COOH, however, is only incorporated into hair in very low concentrations. Detection of the metabolite is therefore analytically very challenging, and as a consequence, in most cases only THC is monitored with respect to the SoHT cut-off value of 0.05 ng/mg.[20] THC detected during hair analysis, however, can also originate from external contamination.[3] Preliminary experiments by Pichini *et al*. show promising results regarding the detection of a phase II metabolite (THC-COOH-glucuronide), which might be an alternative for the detection of THC-COOH.[24] Different studies show that cut-off values and metabolite detection are not always sufficient to distinguish between exposure to drugs and actual drug use.[3,7] As an additional measure, decontamination washes are recommended to remove external contamination from the hair surface. An effective wash protocol can prevent false-positive results, but too thorough washings, on the other hand, can remove incorporated compounds and yield false-negative analysis results.[1,2] Tsanaclis *et al*. describe the use of the ratio between wash residues and drug concentrations detected in washed hair as a supplementary step for the assessment of hair analysis results. A ratio below 0.1 most likely indicates drug use, ratios between 0.1 and 0.5 indicate possible drug use and when a ratio above 0.5 is found, most of the detected drugs originate from external contamination.[3,25]

Over the past 25 years, several papers have been published related to the removal of external contamination from hair samples.[4,5,8,9,11-13,26-38] Most of these studies focus on contamination caused by exposure to cocaine, cocaine solution or cocaine smoke.[4,5,8,9,11,26-36] An extensive protocol, initially described by Baumgartner and Hill,[26] consisting of an isopropanol wash followed by several phosphate buffer washes[8,9,27,28] was found to be adequate for the removal of cocaine contamination, but less successful results have also been reported [5]. Other tested protocols consisted of methanol,[5,11,30,33,34] dichloromethane,[29,31,32] ethanol[4,5], or detergent washes[35,36], but the results thereof vary. Fewer studies have been published evaluating cannabis contamination and the removal thereof.[12,13,37-39] Blank hair has been exposed to hashish or marijuana smoke, and afterwards decontaminated using different protocols. Detergent and water washes were found to be insufficient to remove cannabinoid contamination.[12,13,37] Organic solvents, like dichloromethane, methanol and petroleum ether, appear to have better decontamination properties and removed all, or almost all, contamination caused by cannabis smoke[12,13] or soaking in ethanolic cannabinoid solutions.[37] One study describes external contamination caused by soaking blank hair into an aqueous synthetic cannabinoid solution. Repeated washing with methanol, water and again methanol did not remove this contamination.[38]

Despite the great efforts made in these studies, most of them did not follow the recommendations of the SoHT, which state that a decontamination protocol should consist of, at least, an organic and an aqueous wash.[20] In addition, in the present studies[12,13,37-39] only a limited range of decontamination solvents was investigated, no tests drug user hair samples were performed or only the amount of drugs of abuse present in wash solvents was evaluated instead of the amount still present on hair. In the majority of the recent studies investigating (synthetic) cannabinoid contamination,[14-19] a decontamination protocol consisting of, consecutively, water, acetone and petroleum ether wash steps was used. Since the focus of these studies was on identifying the source of contamination, no evaluation of the used decontamination protocol was included.[14-19] Thus, it can be stated that no general consensus has been reached regarding the best protocol to decontaminate hair samples, especially for cannabinoids. Therefore there is a clear need for an extensive evaluation of the effect of different decontamination protocols for external cannabinoids, mainly THC, originating from different sources,[40] as well as the effect of optimized decontamination protocols on internally incorporated THC in drug users' hair. The main goal of the current study was to develop an effective decontamination protocol, which removes external contamination but does not influence endogenously incorporated compounds. Cannabinoid concentrations, with a focus on THC, in hair samples and wash solvents were monitored using liquid chromatography tandem mass spectrometry (LC–MS/MS) and hair structures were examined using scanning electron microscopy (SEM). Please note that in the context of contamination, THC-COOH was not considered since this is not an external contaminant.

Materials and Methods

Chemicals. Methanol (HPLC gradient grade) was purchased from J.T. Baker Chemicals (Deventer, The Netherlands). Sodium hydroxide, formic acid and acetic acid were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Acetonitrile, *n*-hexane and ethyl acetate were purchased from Actu-All Chemicals (Oss, The Netherlands) and were of Ultra LC–

MS (acetonitrile) or HPLC (*n*-hexane and ethyl acetate) grade. Ethanol was purchased from VWR International (Amsterdam, The Netherlands) and was of analytical grade. Chloroform, petroleum ether (bp 40–60 °C), sodium dodecyl sulfate (SDS) and Tween 20 were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands) and were of analytical grade (chloroform and petroleum ether). Dichloromethane was purchased from Acros Organics (Geel, Belgium) and was of HPLC grade. Purified water was from a Milli-Q system (Millipore, Bedford, MA, USA). The commercial shampoo used was Head & Shoulders Classic (Procter & Gamble, Weybridge, United Kingdom). Van Nelle tobacco (Rotterdam, The Netherlands) was used to mix with cannabis for the smoke experiments. Medicinal cannabis (cannabis flos, variety Bedica; contains approximately 14% THC and 15.6% THCA-A) was purchased from Bedrocan BV (Veendam, The Netherlands). The THC and THC-d3 standards were from Lipomed (Arlesheim, Switzerland) and Cerilliant Corporations (Round Rock, TX, USA), respectively. Δ^9-Tetrahydrocannabinolic acid (THCA-A), THC-COOH, cannabidiol (CBD) and cannabinol (CBN) standards were from Sigma (St. Louis, MO, USA).

Hair samples. Blank hair samples with different hair colors (blond, brown and black) used for contamination experiments were collected from more than 30 non-cannabis users. LC–MS/MS analysis with a limit of detection (LOD) of, respectively, 0.02, 0.06, 0.003, 0.03 and 0.02 ng/mg was used to check that the hair did not contain traces of THC, THC-COOH, THCA-A, CBD or CBN. Cannabis user hair samples (blond and brown) were anonymously collected from two chronic cannabis users stating that they used cannabis multiple times per week during the past 12 months. All the hair of one drug user was collected and over 50 individual locks of hair from all regions of the scalp were used for the presented study. Five individual locks of hair were collected from the second drug user. The hair was collected in Wageningen (The Netherlands) using the forensic hair collection protocol as recommended by the SoHT[20] and informed consent was obtained from each volunteer.

Contamination through soaking. For initial decontamination experiments, 1 g of cannabis plant material was extracted with 50 mL methanol for 20 min under continuous stirring. The resulting extract was filtered and diluted 200 times using water:methanol (80:20) yielding a concentration of 0.3 ppm THC. 2 g blank hair was soaked in 500 mL of the diluted cannabis extract for 60 min and afterwards left to dry for 2h.

Contamination by smoke. The contamination of hair by cannabis smoke was based on the method described by Thorspecken *et al*.:[13] blank hair, as such or first soaked for 15 min in

Milli-Q water to investigate the effect of moisture, or drug user hair samples were placed on a steel mesh in a 6 L desiccator to expose the hair to smoke in all directions. The desiccator was then evacuated for 5 min using a water jet pump. A cigarette, consisting of approximately 1 g tobacco and varying amounts of cannabis (20 and 75 mg for, respectively, low and high THC levels), was made using a tube-filling machine (Mascotte, Eindhoven, The Netherlands) and placed on the inlet of the desiccator. The cigarette was lighted while opening the inlet, enabling the cigarette to burn and the resulting smoke to enter the desiccator (Figure S1A). After the cigarette had burned, the desiccator was closed and the hairs remained in the smoke for 30 min. For high contamination levels, this procedure was then repeated.

Contamination by indirect contact. To mimic cannabis contamination by hand-to-hair contact, medicinal cannabis was rubbed between either gloves or bare hands for 2 min. For contamination, 10 or 75 mg of cannabis material was used. The contaminated gloves/hands were then used to handle blank hair, as such or first soaked for 15 min in Milli-Q water to investigate the effect of moisture, or drug user hair samples for 2 min to transfer cannabinoids from the gloves/hands to the hair samples (Figure S1B). To create samples with reproducible low levels of contamination, a slightly adapted protocol was used. 15 mg cannabis material was rubbed between gloves for 2 min, after which one of the gloves was removed and replaced by a clean glove. The contaminated and clean glove were rubbed together. After 2 min, hair was handled with these gloves for another 2 min.

Decontamination experiments. Before decontamination, hair samples were divided in sub-samples of approximately 60 mg (variation in length 3–6 cm). The sub-samples were transferred to a 15 mL glass test tube and 5.0 mL of the decontamination solvent were added. After vortexing for 10 s and ensuring that all the hairs were in the solvent, the test tubes were shaken for 15 min at 100 rpm. The hair samples were then removed from the test tube using tweezers and placed on paper to dry in a fume hood. When applying sequential decontamination steps, hairs were left to dry for 15 min after a methanol wash step. When SDS was the final wash step in the sequence, hairs were rinsed with Milli-Q water to remove the SDS from the hair and then left to dry for 2 h. Note that rinsing with Milli-Q without drying took place in between an SDS and a methanol wash sequence. Methanol wash solvents were stored for LC–MS/MS analysis.

LC–MS/MS sample preparation. A previous described sample preparation method was used:[41] approximately 60 mg of hair sample was cut using scissors into small pieces and transferred into a 15 mL glass test tube. After addition of 5.0 µL 1 ppm THC-d3 in methanol as internal standard and 1.0 mL 1 M NaOH, the hairs were incubated for 10 min at 95 °C. Next, the samples were cooled to room temperature and the pH was adjusted to 2 using concentrated acetic acid. Liquid–liquid extraction was performed in the test tube using 3.0 mL of *n*-hexane/ethyl acetate (90:10). The organic phase was separated using a Pasteur pipette, evaporated under a gentle N_2 flow in a water bath at 40 °C and reconstituted in 100 µL methanol. Afterwards, a 5.0 µL aliquot was injected into the LC–MS/MS system.

Methanol wash solvents were analyzed as follows: 5.0 µL 1 ppm THC-d3 in methanol was added as internal standard to 5 mL wash solvent in a 15 mL glass test tube. After evaporation under a gentle N_2 flow in a water bath at 40 °C, the samples were reconstituted in 100 µL methanol and 5.0 µL was injected into the LC–MS/MS system.

LC–MS/MS method. The cannabinoid concentration in hair samples and wash solvents was determined using an in-house validated and externally benchmarked LC–MS/MS method:[41] an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) was coupled to a Micromass Quattro Ultima Platinum (Waters) mass spectrometer. Positive electrospray ionization was used for THC, THC-d3, THC-COOH, CBD and CBN under the following conditions: capillary voltage, +3.0 kV; cone voltage, 35 V; source temperature, 120 °C; desolvation temperature, 450 °C; nitrogen cone gas flow rate, 150 L/h; nitrogen desolvation gas flow rate, 600 L/h; collision energy, 30 eV. For the analysis of THCA-A, negative electrospray ionization was used with a capillary voltage of −2.7 kV and a cone voltage of −35 V. Multiple reaction monitoring mode was used with the following transitions: *m/z* 315.3 to *m/z* 123.1, 193.2 and 259.2 for THC, and *m/z* 318.3 to *m/z* 196.2 and 262.3 for THC-d3. THCA-A, THC-COOH, CBD and CBN were analyzed using the following transitions: *m/z* 357.3 to *m/z* 179.2, 191.1, 245.1 and 313.3 for THCA-A, *m/z* 345.3 to *m/z* 119.1, 193.1 and 299.3 for THC-COOH, *m/z* 315.3 to *m/z* 123.1, 193.2 and 259.2 for CBD and *m/z* 311.3 to *m/z* 195.2, 223.1 and 241.1 for CBN. Data analysis was performed using MassLynx V4.1 (Waters). Baseline separation for all analytes (Figure S2) was achieved using a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA, USA), at 50 °C. The mobile phases consisted of 5 mM formic acid in water (mobile phase A) and acetonitrile (mobile phase B) and the flow rate was 0.80 mL/min. 2 min isocratic elution with 25% B was followed by a gradient towards 100% B in 7 min. After 2 min at this condition, the system was re-equilibrated for 3 min at 25% B. Quantification was achieved using a matrix-matched calibration curve and internal standard correction. For this purpose, cannabinoid standards were added to blank hair in concentrations ranging from 0.2 to 20 ng/mg hair. The resulting, internal standard corrected, calibration curve was then used for concentration calculations in the hair samples.

Data analysis. Student's *t* test (two-sample assuming unequal variances) was performed using Microsoft Excel and used to assess significance of results from different wash solvents and protocols.

Scanning electron microscopy. SEM was used to visualize the effect of the contamination and decontamination steps on the hair structure. The hair samples were attached to a holder using carbon tape, after which a gold coating was applied. The hair structure was visualized using a JEOL JSM-5600LV scanning electron microscope (JEOL Ltd., Tokyo, Japan). The SEM images presented were obtained using the following settings: acceleration voltage, 25 kV; spot size, 3; magnification, 750×–1300×.

Results and Discussion

To be able to test the decontamination properties of different wash solvents and sequential steps, hair had to be externally contaminated. Two approaches to realistically mimic external cannabinoid contamination were selected; contamination by cannabis smoke and contamination by indirect contact with cannabis plant material. Both scenarios are well possible to cause external contamination in real life situations by passive contact with cannabis smoke or contact with persons who handled cannabis material. Prior to this, initial tests were conducted using blank hair samples, which were soaked in a diluted cannabis extract. Soaking caused more radical and unrealistic contamination, but nevertheless offered a framework for determination of the best strategies to follow during this study. After careful evaluation of the contamination procedures, large batches of hair were contaminated to extensively test different decontamination strategies. A schematic overview of the entire study is shown in Figure S3.

External contamination of blank hair by cannabis smoke

To model the effect of cannabis smoke on hair, blank hair samples were placed in a desiccator, which was next filled with smoke from a cannabis cigarette (Figure S1A).

Where initial tests with 100 mg cannabis added to a 1 g tobacco cigarette led to high contamination of more than 30 ng THC per mg hair, 10 mg cannabis resulted in a contamination level of several nanograms per mg of hair (Table 1) which is comparable to concentrations found in drug user hair (ranging from 0.1 to 7.6 ng/mg[12,21-23]). The contamination levels were consistent with the levels found by Moosmann *et al*.[15] and Thorspecken *et al*.[13], up to 1.7 and 20 ng/mg, respectively. Batches of both dry and wet hair were contaminated simultaneously to investigate the effect of moisture, that is, to mimic hair wetted by rain or by taking a shower. Since no significant difference was found between the batches of dry and wet hair ($P < 0.01$, Figure S4), it was concluded that moisture did not influence the level of external contamination. One of the reasons for this could be that THC is quite hydrophobic and, consequently, its deposition from the cannabis smoke does not benefit from moisture on the hair. In addition, the scenario of a person having fully wet hair coming in contact with cannabis smoke is not realistic in most cases and wet hair was therefore not used in the rest of the study. The homogeneity of the batches of smoke-contaminated hair was determined by analyzing multiple sub-samples originating from one batch. Making cigarettes using a tube-filling machine increased the reproducibility of the content of the cigarettes. The relative standard deviation (RSD) found within a batch ranged from 3 to 27% (Table 1). The RSD between batches made on the same day, but with different hairs and cannabis cigarettes, ranged from 7 to 36% (Table 1). Taking into account that hair is a complicated biological matrix and the deposition of compounds from smoke onto the hair surface is a complex process, it can be concluded that the contamination procedure using cannabis smoke was reproducible enough to be fit for purpose.

External contamination of blank hair by indirect contact with cannabis plant material

Besides contamination originating from smoke, cannabis traces can also be transferred onto the hair by hand-to-hair contact while handling cannabis plant material in general (e.g., while working in a cannabis shop) or by (indirect) contact with persons who handled cannabis material. This was simulated by rubbing cannabis plant material between bare hands or gloves and afterwards handling hair samples with the contaminated hands/gloves. When using 75 mg of cannabis, contamination levels of 46–83 ng THC per mg hair were achieved (Table 1). Since such levels are approximately tenfold higher than the levels observed in drug user hair, most contamination experiments were performed using less cannabis (10 mg). The resulting lower contamination levels (Table 1) were still higher than those found by Moosmann *et al*.[19] in hair of individuals handling cannabis plant material (<0.09 ng/mg), however, their study focused on contamination after rolling a cannabis cigarette instead of the standardized contamination protocol used in this study. As with smoke contamination, also wet hair was contaminated to investigate the effect of moisture. The resulting contamination again did not significantly differ ($P < 0.01$) from the contamination levels on dry hair (Figure S4). Additionally, the effect of wet gloves was explored but this did not result in a significant difference ($P < 0.01$) over the use of dry gloves. Another parameter that could influence the contamination levels is the duration of handling the hair. It was found that handling hair for 1, 5 or 10 min did not change the resulting external contamination, indicating that after a certain time, likely within 1 min, the transfer of cannabinoids to the hair is complete. To ensure that all hairs came in repeated contact with the contaminated hands or gloves and the contamination is as evenly spread over the hair sample as possible, a somewhat longer handling time (2 min) was chosen for the remaining experiments. The homogeneity of a batch of indirectly contaminated hair was found to be fit for purpose (RSD ranging from 3 to 23%), the between batch RSD was between 11 and 30%.

Decontamination performance of aqueous and organic solvents

The batches of contaminated blank hair samples were divided into sub-samples and used to test different solvents regarding their decontamination performance in a single wash step procedure. As organic solvents, acetonitrile, ethyl acetate, methanol, ethanol, chloroform, dichloromethane and petroleum ether 40–60 were considered. Water, of Milli-Q quality for all experiments, and 5% aqueous detergent solutions of SDS (ionic) and Tween 20 (non-ionic), were chosen to test aqueous decontamination properties. Figure 1 shows the normalized THC concentrations left on the hair sub-samples after decontamination with a single wash. The experiments were performed on two different days with three sub-samples for each solvent per day and with different batches of contaminated blank hair. The initial concentrations, respectively, 2.6 and 2.8 ng/mg for smoke and indirect contact contamination, were set to 100%. A clear distinction in decontamination properties was found between aqueous and organic solvents. Aqueous solvents removed 45% or less of the THC contamination originating from smoke, while all organic solvents removed 93% or more, except petroleum ether. THC contamination after indirect contact with cannabis material was for 73% removed using aqueous solvents. Organic solvents removed at least 94%, with the exception of petroleum ether and acetonitrile. Use of a commercial shampoo resulted in decontamination results similar to the SDS and Tween 20 solutions, as expected since the main detergent component in most commercial shampoos is SDS. The same experiment was repeated using higher contamination levels, which resulted in comparable differences between the different solvents (Figure S5). Decontamination of hair samples soaked in diluted cannabis extract yielded similar results (Figure S6). Petroleum ether did not decontaminate as well as described by Strano-Rossi *et al.*,[12] the good performance of methanol as decontamination solvent, however, is in good agreement with the findings of Thorspecken *et al.*[13] The main difference between smoke and indirect contact contamination was that smoke contamination was better removed using organic solvents and indirect contact contamination was relatively better removed with aqueous solvents (Figure 1 and Figure S5). Smoke contamination might be more adhering to the hair due to absorption from the gas phase, and thus making an organic wash solvent necessary for removal, while indirect contact contamination is more likely to be physically deposited on top of the hair surface.

Since no decontaminated sub-sample resulted in a blank measurement, it was concluded that not all external cannabis contamination could be removed from blank hair using a single wash step. As recommended by the SoHT, only multiple decontamination steps with combinations of different solvents constitute a proper decontamination procedure. To limit the number of combinations of decontamination steps, a selection was made from the solvents tested. Although the differences were small, an aqueous solution of SDS was chosen over Tween 20 since the Tween 20 solution gave larger RSDs. Methanol, dichloromethane and chloroform were the organic solvents with the best decontamination properties. Methanol was selected as the preferred organic solvent due to its lower toxicity and environmental impact compared to dichloromethane and chloroform.

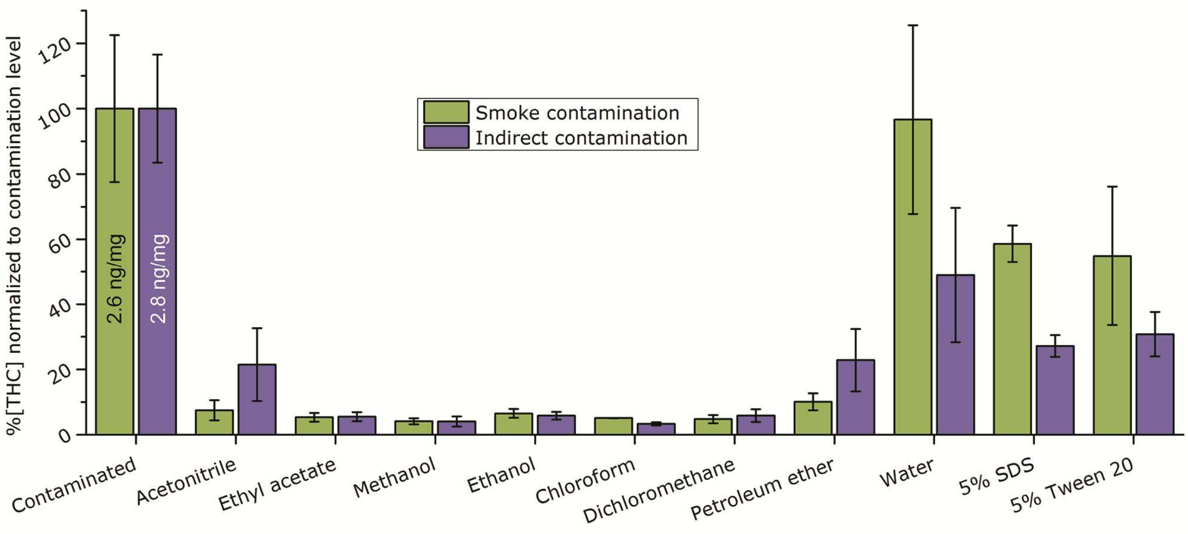

Figure 1. *Normalized THC levels in hair following single wash decontamination after cannabis contamination (n = 6).*

Decontamination using sequential wash steps

As a start, combinations of two or three sequential wash steps using methanol and water were tested on blank hair with low levels of smoke or indirect contact external contamination (Table 1). After decontamination, the THC content in the hair samples was below the LOD of the LC–MS/MS method (0.02 ng/mg) proving that all contamination was removed (Figure 2A). Since the LOD is below the cut-off value for THC of 0.05 ng/mg recommended by the SoHT,[20] the decontaminated samples would yield a negative result. So at low external contamination levels, no differences between sequential washes could be assessed and therefore the experiment was repeated using higher levels of contamination. Using external contaminations of more than 25 ng/mg, i.e. mimicking rare levels, some external THC left on the hair was still detectable. Figure 2B shows the initial sequences tested, including a single wash step with water or methanol. The resulting graph clearly shows that starting with a methanol wash step results in better removal of cannabis contamination compared to starting with an aqueous wash, most likely caused by the better solubility of cannabinoids in organic solvents. Drying after an aqueous wash step had a negative effect on the total decontamination performance of a sequence: an aqueous wash immediately followed by a methanol wash removed more contamination compared to the same sequence with a 2 h drying step in between, even though the difference was not significant. To decide whether or not the total decontamination procedure could be improved through use of a detergent solution, two and three step protocols were tested with both water and 5% SDS solution. The results showed a significant difference ($P < 0.05$) between the use of water and SDS solution for the protocols consisting of two wash steps (Figure 2C), as could be anticipated from the results using a single wash step (Figure 1). The improved decontamination after addition of a detergent to water was to be expected for a compound with low water solubility like THC. The difference between water and SDS was not found for soaked hair samples, for those samples all the tested protocols removed only ~90% of the contamination (Figure S7). This is most likely because soaking results in more severe contamination, which is harder to remove, as was also noted by Saito *et al*. when they soaked hair in a solution containing synthetic cannabinoids.[38] From the results in Figure 2B and 2C, three sequential wash protocols were chosen for further testing on drug user hair samples: (1) methanol, SDS; (2) methanol, SDS, methanol and (3) methanol, methanol. The two protocols using both methanol and SDS washes, follow the SoHT guidelines.[20] The methanol, methanol protocol does not follow the recommendation, but performed equally well. Using any of these three sequential protocols, all the realistic low level external contamination is removed, yielding a negative hair analysis result, and more than 99% of the unrealistic high level contamination was removed. Next, the three protocols were further tested on both uncontaminated and externally contaminated drug user hair samples, see below.

Table 1. *THC levels after both low and high contamination of blank hair samples using the smoke or indirect contact contamination procedure and the corresponding within and between batch RSDs.*

		[THC] after contamination (ng/mg)	RSD within batch	RSD between batch
Smoke contamination	Low	2.2–3.8	3–27%	7–36%
	High	32–52		
Indirect contact contamination	Low	2.6–3.1	3–23%	11–30%
	High	46–83		

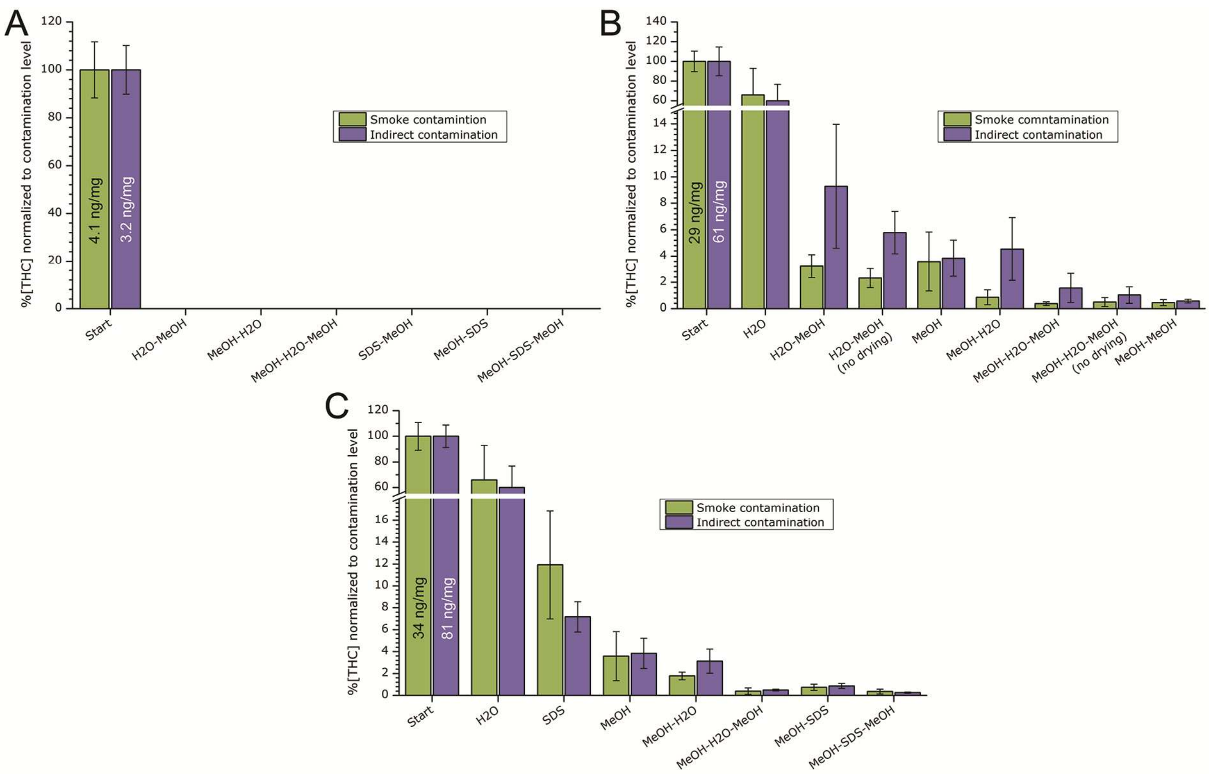

Figure 2. *Normalized THC levels in hair following A) sequential wash protocols after low level cannabis contamination (n = 6); B) sequential wash protocols using Milli- Q water and methanol after high level cannabis contamination (n = 6); and C) sequential wash protocols using Milli-Q water or 5% aqueous SDS solution in combination with methanol after high level cannabis contamination (n = 6).*

SEM evaluation of contaminated and decontaminated hair

SEM imaging was used to determine whether or not the outside layer of the hair, the cuticle, is affected by the (de)contamination procedures. Damage to the hair cuticle could affect the decontamination procedures by either the possibility that contamination can reach deeper regions of the hair, or by a decontamination solvent removing endogenously incorporated compounds. The need for examination of the hair cuticle using SEM has been previously suggested by Stout *et al*.[42] Figure 3A shows a SEM image of untreated hair. The scale-like structure of the cuticle is clearly visible, proving that the SEM used for these experiments had enough magnification power to be suitable for investigating the effect of treatments. After smoke contamination small irregularities were visible (Figure 3B), however, these were only minor and could sometimes also be observed on untreated hair. Overall, the cuticle was unharmed after smoke contamination. Indirect contact contaminated hair showed a very different image: several irregularities could be seen over the whole length of the indirectly contaminated hairs (Figure 3C) compared to untreated hair. The irregularities seemed to be damaged parts of the cuticle caused by handling the hair (some of the cuticle scales seemed to have been lifted) or cannabis particles deposited onto the hair.

Hair samples were also imaged after exposure to the selected decontamination protocols to detect damage caused by the wash steps. SEM images of blank hair before and after a 15 min methanol wash do not show significant differences (Figure 3D). Hair samples washed in aqueous SDS solution, however, showed some lifted cuticle scales (Figure 3E). The research of Stout *et al*.[42] showed similar SEM images: no damage after washing with methanol and some possible cuticle damage when using an aqueous phosphate buffer. This may cause removal of incorporated drugs during decontamination steps, and thus yield false negative results. Investigation of the effect of the decontamination protocols on drug user hair was therefore crucial. After decontamination with methanol and aqueous SDS solution, some of the observed irregularities on hair samples that came indirectly into contact with cannabis plant material (Figure 3C) had been removed, but the hair surface did not completely return to the state of untreated hair. This is another indication that artefacts on the hair surface are not only small pieces of cannabis plant material, but also damage to the hair caused by rubbing the hair for several minutes during deliberate contamination. In real life situations, hair will be handled for a shorter period and less rigidly, so we consider this as a worst-case scenario. Note that the results in Figures 1 and 2 and Figure S5 show that any hair damage had no influence on the decontamination efficiency.

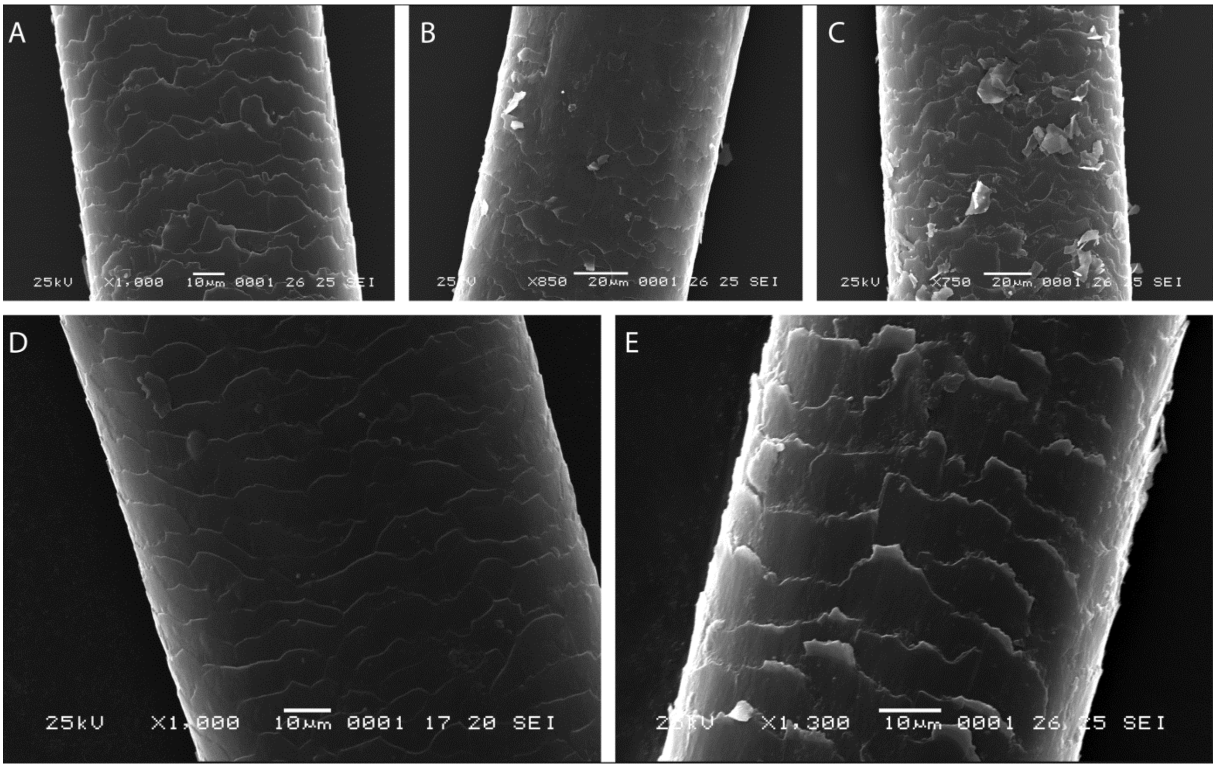

Figure 3. *SEM images of A) untreated hair; B) smoke-contaminated hair; C) indirectly contaminated hair; D) hair after decontamination with methanol; and E) hair after decontamination with aqueous SDS.*

Performance of selected decontamination protocols on drug user hair samples

The three best performing sequential decontamination protocols: (1) methanol, SDS; (2) methanol, SDS, methanol and (3) methanol, methanol were thoroughly tested on both drug users' hair as such, and after additional external contamination. The contamination and decontamination experiments were initially completed using over 50 individual locks of hair sampled from one chronic cannabis user which contained THC levels ranging from 0.4 to 1.6 ng/mg according to LC–MS/MS analysis, a variation in good accordance with the recent findings of Dussy *et al*.[43] reporting variations up to 62% in ethyl glucuronide and caffeine concentrations between locks of hair originating from the same individual. Decontamination of the drug user hair samples as such resulted in a loss of ~34% of the total THC content: most likely, also the drug users' hair contained external contamination originating from smoke during usage and/or from hand-to-hair contact of the user after handling cannabis. No significant difference ($P < 0.05$) was found between the three tested sequential decontamination protocols regarding the removed amount of THC, indicating that the three protocols worked equally well on drug user hair samples. To ensure that no contamination was left on the hair and that no incorporated THC was removed, a similar experiment was performed using multiple sequential methanol washes (1 up to 5). The THC content did not decrease any further when using more than two sequential methanol washes indicating that all the contamination was removed after the second methanol wash step and no incorporated THC was removed (Figure S8). Furthermore, only the first two methanol wash solutions contained THC according to LC–MS/MS analysis, corresponding to, respectively, 0.13 and 0.09 ng/mg as expressed in hair levels.

Next, hair samples from the same chronic cannabis user were additionally contaminated using the smoke or the indirect contact protocol, resulting in total THC concentrations of 2.5–5.8 ng/mg and 4.4–5.8 ng/mg for smoke and indirect contact contamination, respectively. After decontamination using any of the three sequential protocols, the resulting THC concentrations did not significantly differ ($P < 0.05$) from the concentrations in the originally decontaminated drug user hair samples (Figure 4). Overall, the mass balance using the detected THC concentrations in the decontaminated hair samples and the two methanol washes (note that SDS wash solvents could not be analyzed using LC–MS/MS due to the high detergent content) ranged between 83% and 118%. Even when applying less realistic very high external contamination levels on drug user hair, all smoke contamination was removed after decontamination. For high level indirect contact contamination, however, not all external contamination was successfully removed (Figure S9). Approximately 5% of the added external contamination remained on the drug user hair, unlike blank hair samples where less than 1% of the high level external contamination remained. SEM imaging of drug user hair, before and after both contamination and decontamination, did not show any differences compared to the images obtained using blank hair samples. The incomplete removal of high level indirect contact contamination could be an anomaly of the hair of this particular drug user. For that reason, the methanol, methanol decontamination protocol was tested on an additional externally contaminated hair sample from a different drug user, and no contamination remained on the hair after washing. Unfortunately, not enough drug user hair samples were available to repeat all the experiments with hair from the second drug user. Nonetheless, based on the extensive testing with blank hair and over 50 locks of drug users hair, it is expected that the proposed decontamination procedures can be used at forensic end-user laboratories after minor in-house validation. It has to be considered that if a forensic sample contains unrealistically high levels of external cannabis contamination, such as used in the high indirect contact contamination experiments, there is a severe chance that not everything is removed during decontamination and a false positive result could be obtained. When evaluating the THC concentration in wash solvents from all contaminated samples, wash-to-hair ratios well above 0.5 were found. In this case, the high ratios would, following the criteria described by Tsanaclis et al.,[3,25] point out that most of the drugs originate from external contamination and drug use is questionable. Although the majority of the THC found was indeed coming from external contamination, drug use was in this case not questionable. High contamination could thus mask drug use when following the wash-to-hair ratio criteria, which shows once more that careful interpretation of hair analysis results is crucial.

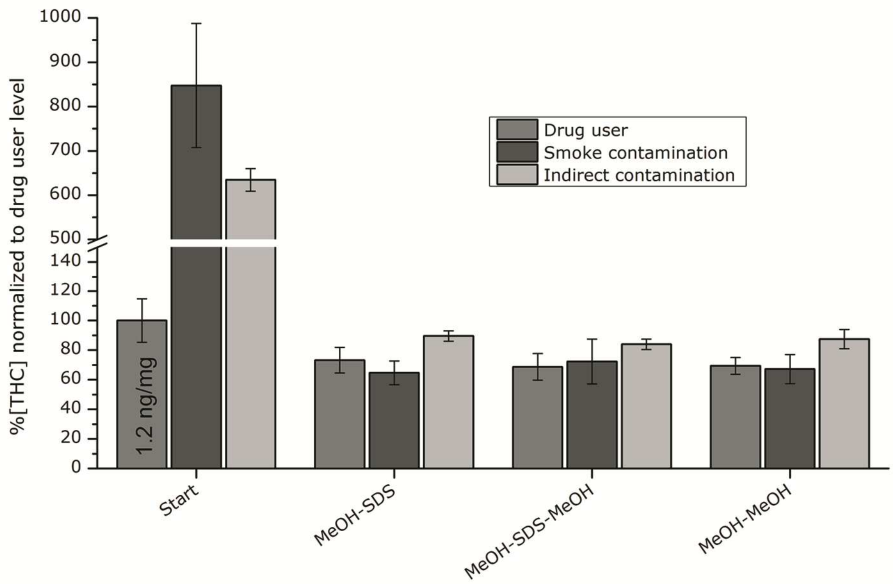

Figure 4. *Normalized THC levels in drug user hair, both without and with additional contamination, following decontamination using the selected protocols (n = 6).*

Effect of decontamination protocols on other cannabinoids

Even though this research focused on THC contamination, other cannabinoids were monitored as well. CBD can be present in high levels in cannabis plant material, however, in the medicinal cannabis used in this study, the CBD content was below 1%. The main metabolite of THC, THC-COOH, was unfortunately not present in LC–MS/MS detectable amounts in the drug user hair samples (LOD for THC-COOH was 0.06 ng/mg) and the effect of the decontamination protocols on THC-COOH could therefore not be monitored. CBN and THCA-A were present in the cannabis material in detectable concentrations and could therefore be followed using LC–

MS/MS. Especially THCA-A has been given quite some recent attention since it is suggested as a marker for external contamination following the handling of cannabis plant material.[15,16,19,44] While indeed high levels of THCA-A contamination were detected after indirect contact contamination only, both THCA-A and CBN followed the same trend as THC after contamination and subsequent decontamination (Figures S10 and S11) and therefore it can be stated that the results discussed in this paper not only apply to THC but also to other, natural, cannabinoids. The effectiveness of the decontamination protocols on synthetic cannabinoids, which are used more and more often, is unknown, but compounds with similar physicochemical properties as THC will probably exhibit comparable behavior during decontamination.

Conclusions

After extensive testing of both single and sequential decontamination protocols, three sequential wash protocols were found to perform equally well regarding the removal of external cannabis contamination originating from smoke or indirect contact: (1) methanol, SDS; (2) methanol, SDS, methanol and (3) methanol, methanol. These protocols removed all realistic contamination levels and did not remove incorporated THC, which is in line with the findings of Baumgartner *et al*. that shampooing has no effect on incorporated drugs in hair.[45] Since no significant differences were found between the performance of the three protocols and to minimize the risk of removing incorporated compounds and save time and chemicals, it is advised to use a protocol consisting of two wash steps. Concluding, taking the recommendations of the SoHT into account, a decontamination procedure using a single methanol wash followed by a single aqueous SDS solution wash is suggested as the preferred decontamination protocol to remove cannabis contamination.

Even though recent publications question the use of decontamination protocols due to incomplete removal of contamination or diffusion into the hair matrix,[19,38] this study provides scientific evidence that decontamination can be a reliable tool to remove possible external contamination originating from cannabis smoke or indirect contact with cannabis plant material, taking into account that caution is advised for samples with extremely high cannabis contamination. Undoubtedly, metabolite detection remains the best approach to indisputably prove drug use, but is still a big analytical challenge given the low incorporation rates of THC metabolites.

Acknowledgments

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Supporting information Chapter 2

Figure S1. *External contamination procedures: A) contamination by cannabis smoke, and B) contamination by indirect contact with cannabis plant material.*

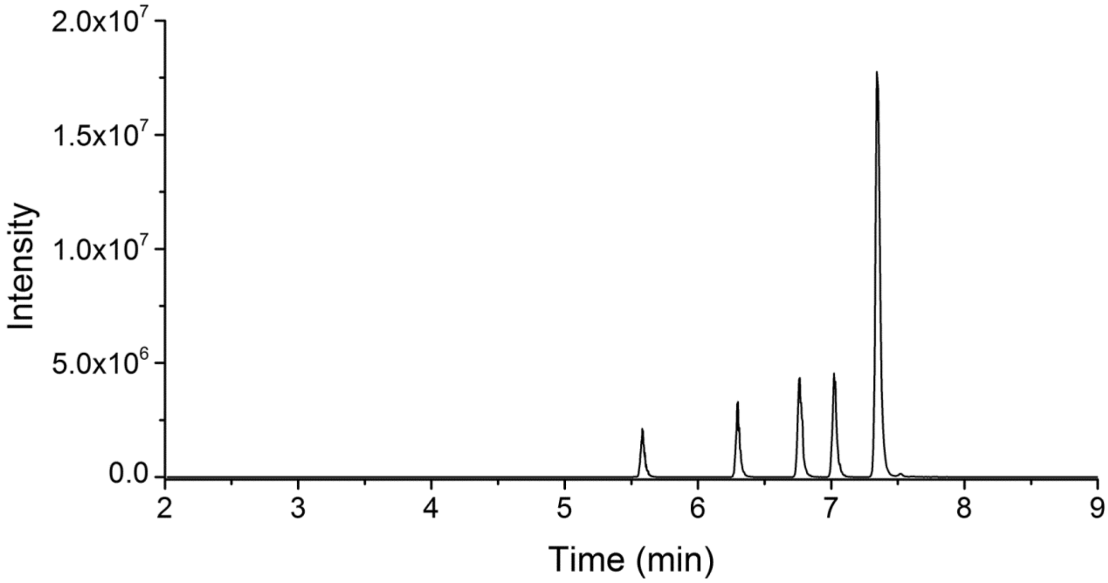

Figure S2. *LC-MS/MS chromatogram of a mixture containing 10 ppm of THC-COOH, CBD, CBN, THC and THCA-A.*

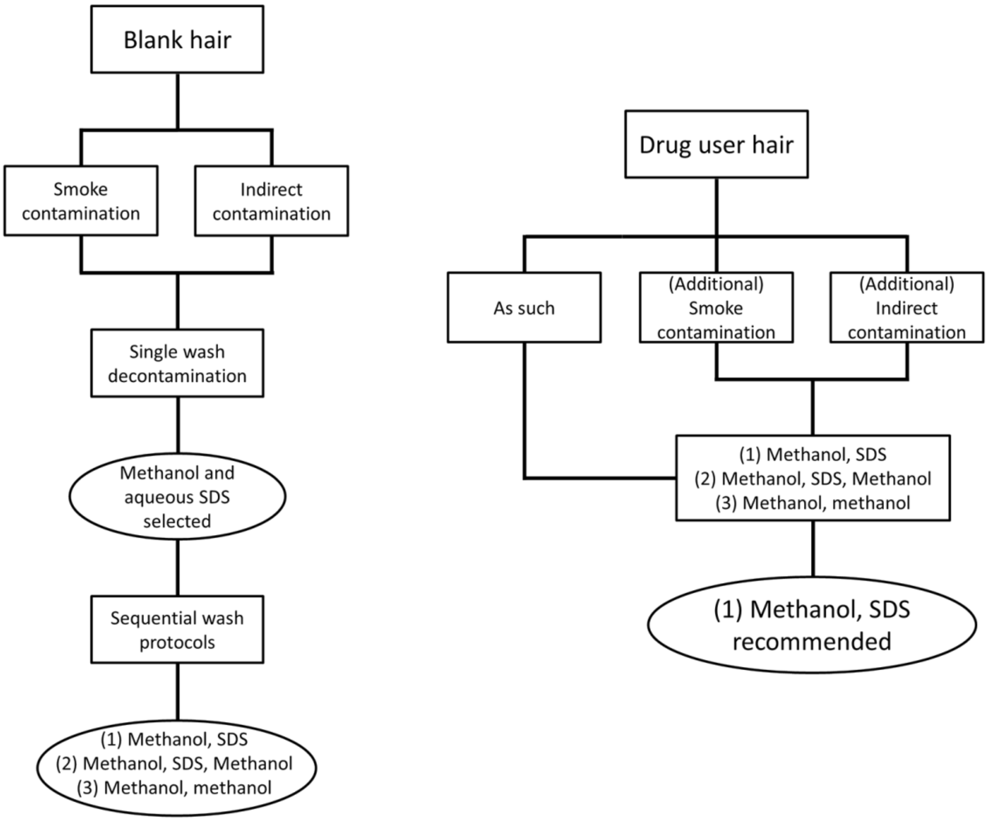

Figure S3. *Schematic representation of the contamination and decontamination study, performed on both blank and drug user hair.*

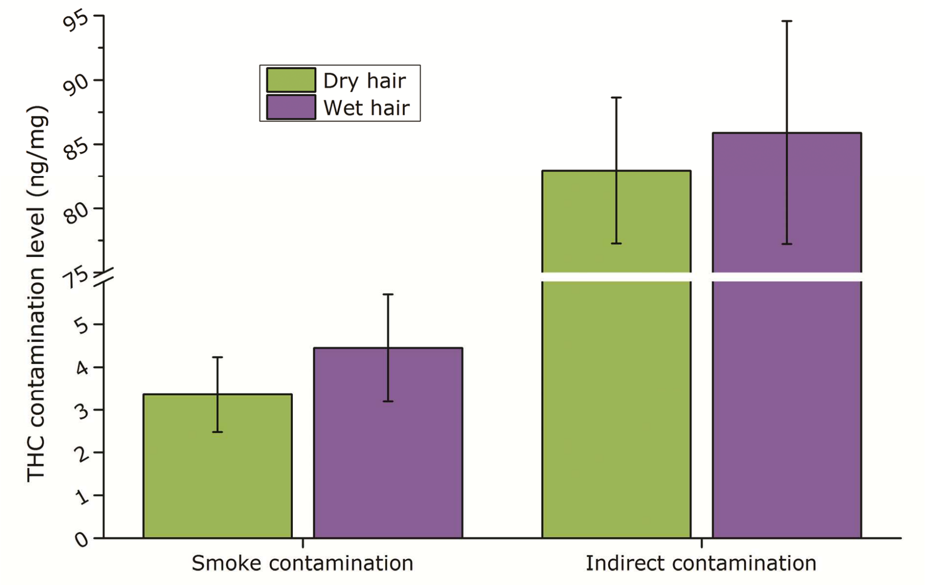

Figure S4. *Effect of moisture on the THC contamination levels in blank hair using the smoke and indirect contact contamination protocols (n=6).*

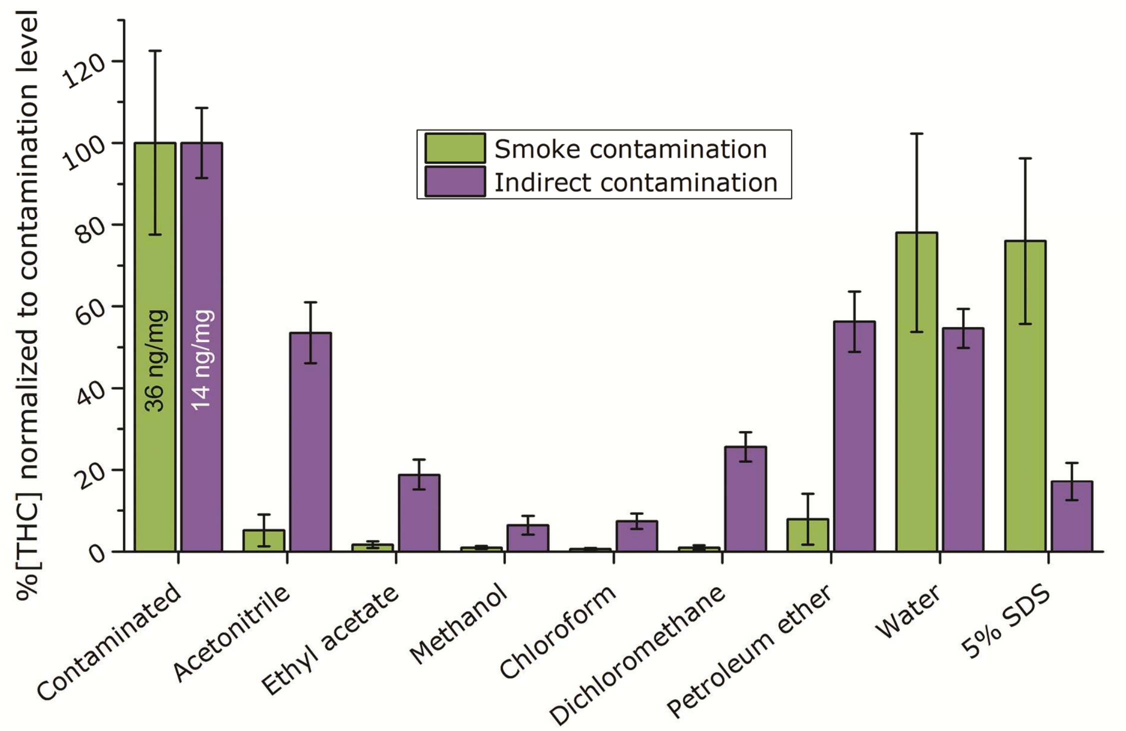

Figure S5. *Normalized THC levels in blank hair following single wash decontamination after high level cannabis contamination (n=6).*

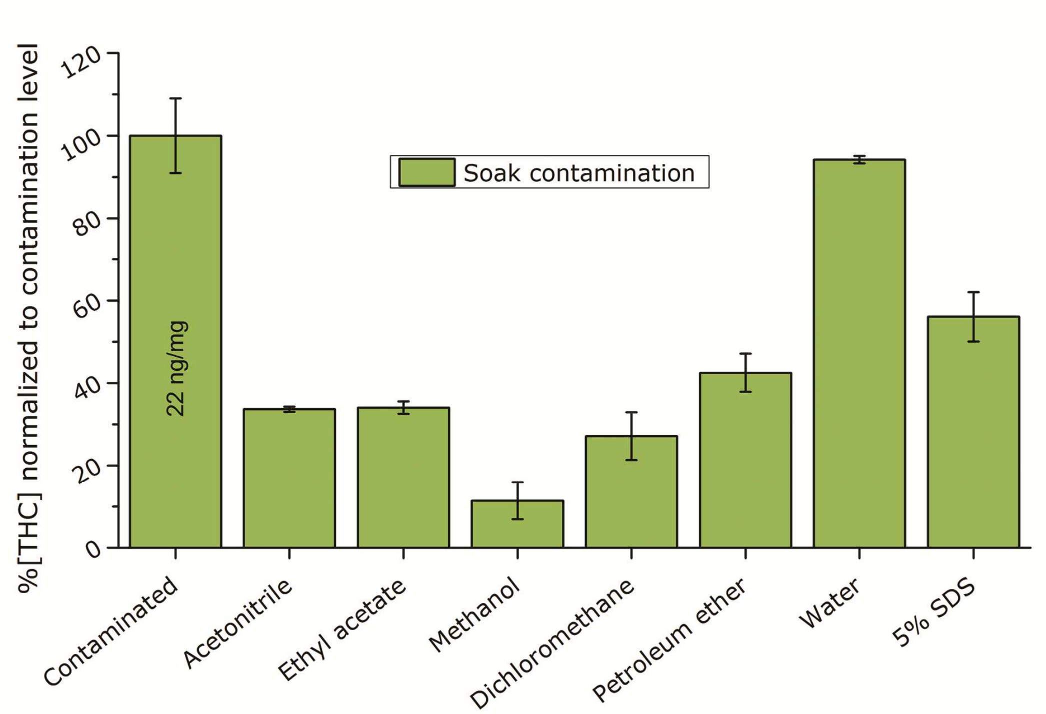

Figure S6. *Normalized THC levels in blank hair following single wash decontamination after cannabis contamination by soaking (n=3).*

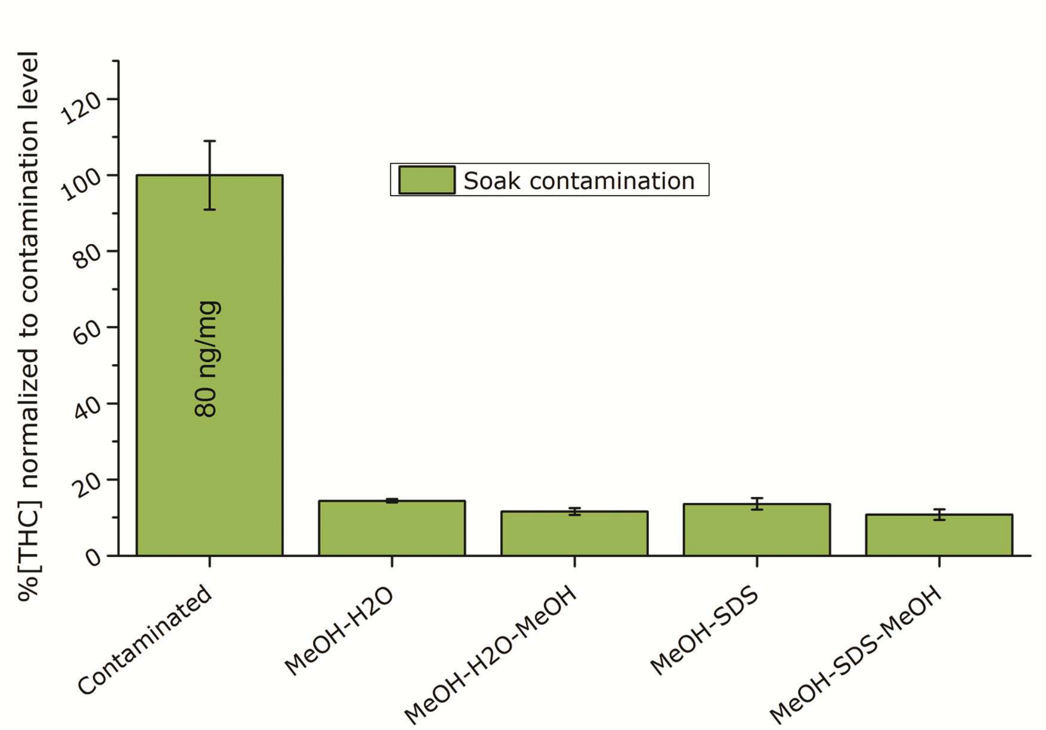

Figure S7. *Normalized THC levels in blank hair following sequential wash protocols after cannabis contamination by soaking (n=3).*

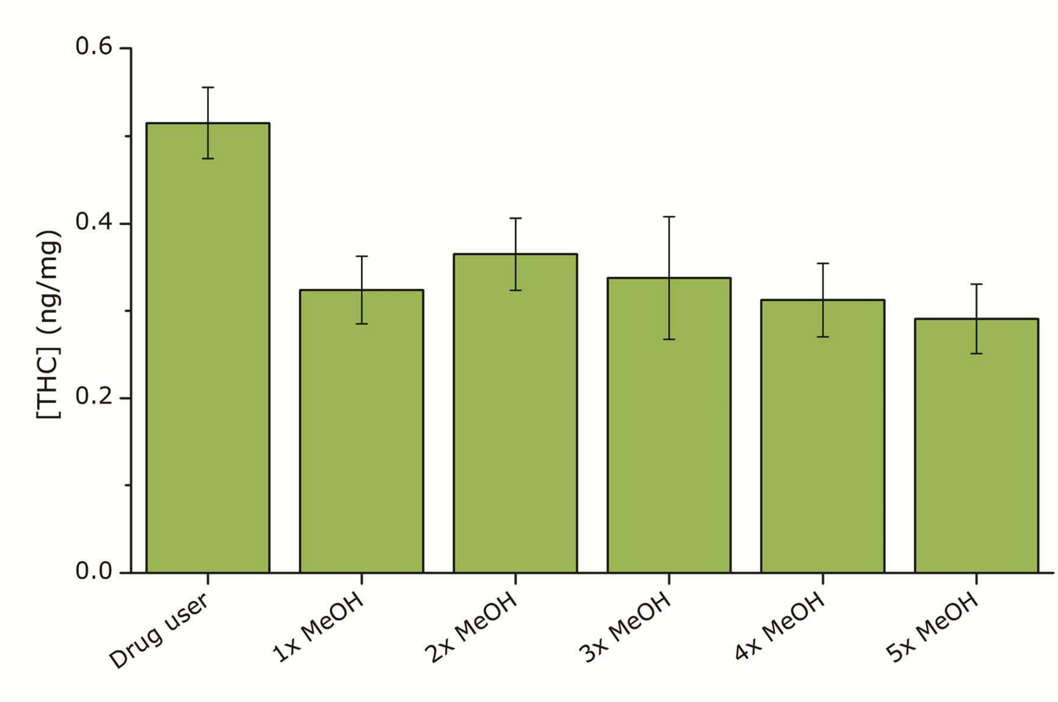

Figure S8. *Normalized THC levels in drug user hair following multiple, sequential, methanol wash steps (n=6).*

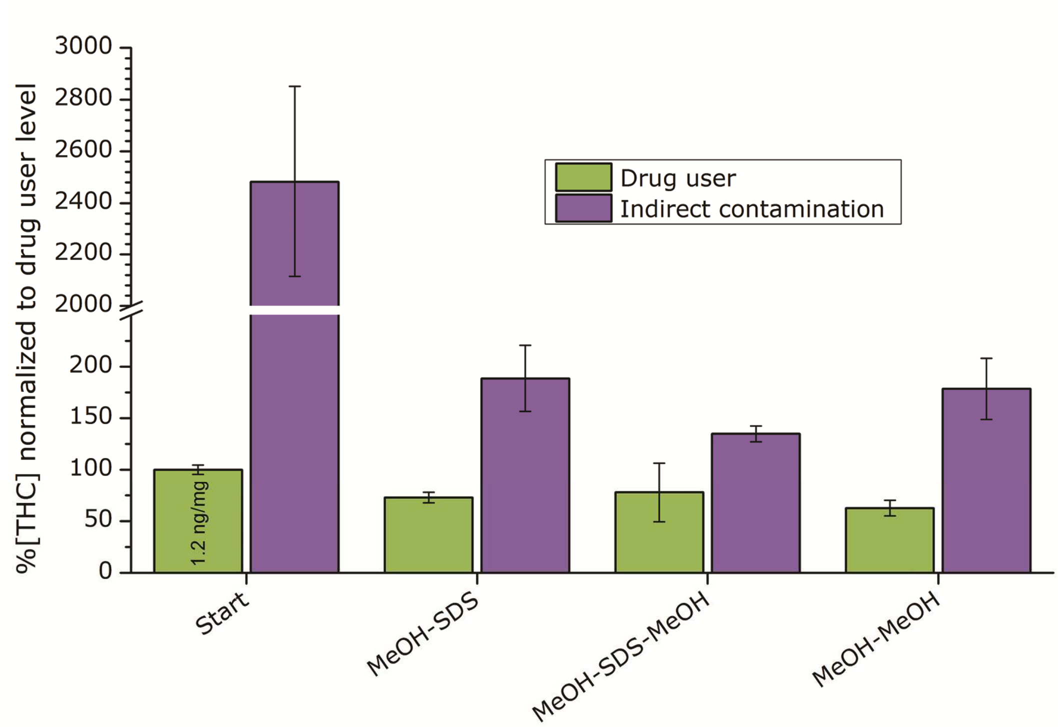

Figure S9. *Normalized THC levels in drug user hair, both without and with additional high level indirect contact contamination, following decontamination using the selected protocols (n=6).*

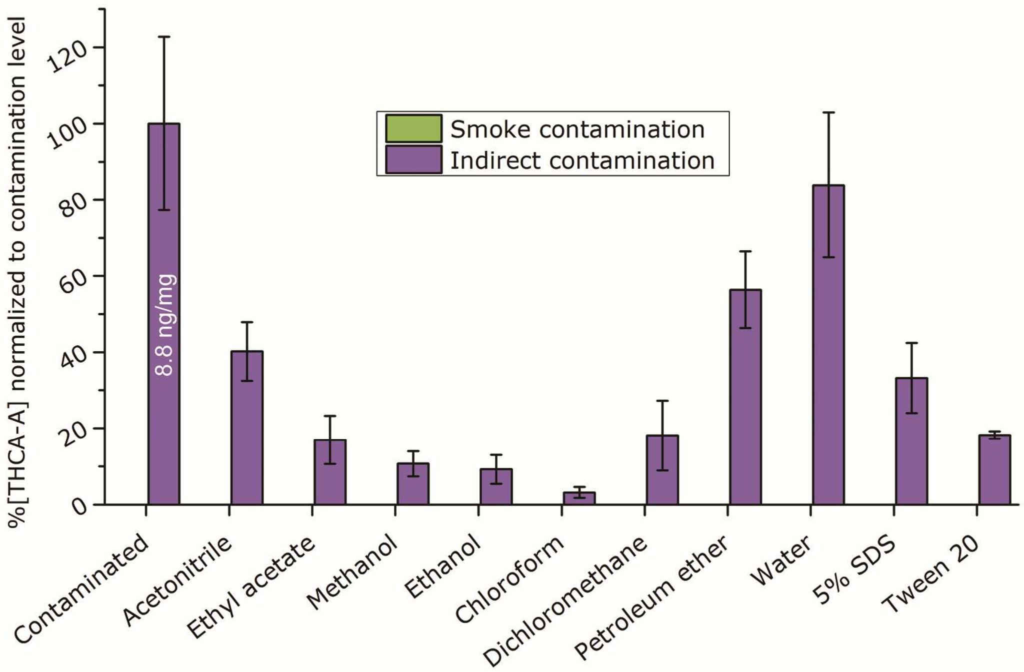

Figure S10. *Normalized THCA-A levels in blank hair following single wash decontamination after cannabis contamination (n=6). Note that smoke contamination did not result in detectable THCA-A concentrations in blank hair.*

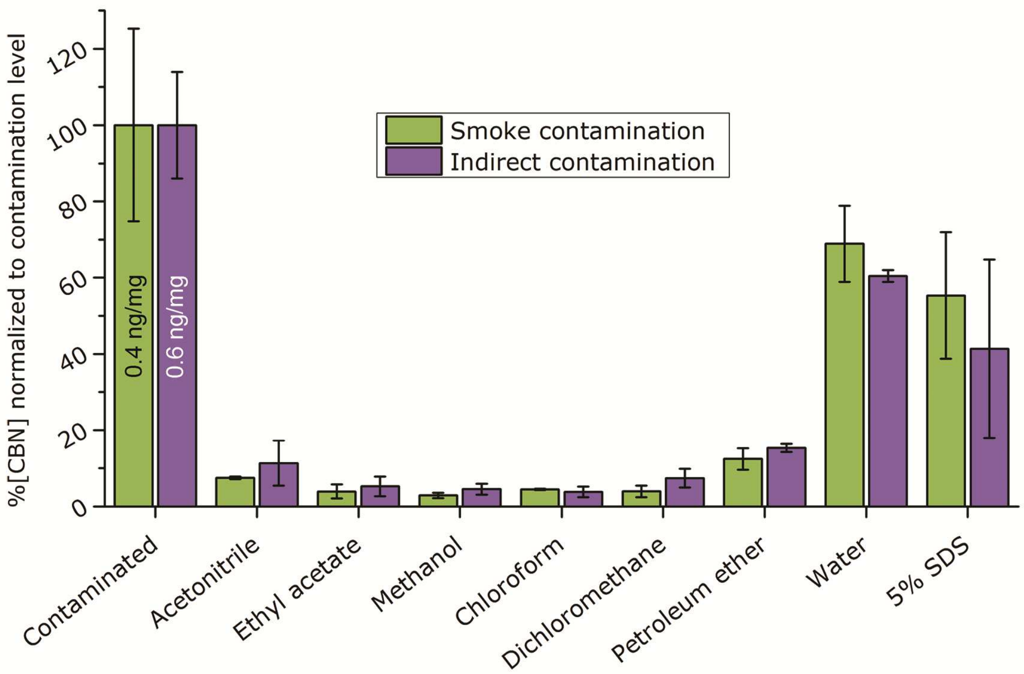

Figure S11. *Normalized CBN levels in blank hair following single wash decontamination after cannabis contamination (n=6).*

Chapter 3

Rapid Analysis of Δ^9-Tetrahydrocannabinol in Hair using Direct Analysis in Real Time Ambient Ionization Orbitrap Mass Spectrometry

This chapter was published as:

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Abstract

RATIONALE: Forensic hair analysis methods are laborious, time-consuming and provide only a rough retrospective estimate of the time of drug intake. Recently, hair imaging methods using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) were reported, but these methods require the application of MALDI matrix and are performed under vacuum. Direct analysis of entire locks of hair without any sample pretreatment and with improved spatial resolution would thus address a need.

METHODS: Hair samples were attached to stainless steel mesh screens and scanned in the X-direction using direct analysis in real time (DART) ambient ionization orbitrap MS. The DART gas temperature and the accuracy of the probed hair zone were optimized using Δ^9-tetrahydrocannabinol (THC) as a model compound. Since external contamination is a major issue in forensic hair analysis, sub-samples were measured before and after dichloromethane decontamination.

RESULTS: The relative intensity of the THC signal in spiked blank hair versus that of quinine as the internal standard showed good reproducibility (26% RSD) and linearity of the method (R^2 = 0.991). With the DART hair scan THC could be detected in hair samples from different chronic cannabis users. The presence of THC was confirmed by quantitative liquid chromatography/tandem mass spectrometry. Zones with different THC content could be clearly distinguished, indicating that the method might be used for retrospective timeline assessments. Detection of THC in decontaminated drug user hair showed that the DART hair scan not only probes THC on the surface of hair, but penetrates deeply enough to measure incorporated THC.

CONCLUSIONS: A new approach in forensic hair analysis has been developed by probing complete locks of hair using DART-MS. Longitudinal scanning enables detection of incorporated compounds and can be used as pre-screening for THC without sample preparation. The method could also be adjusted for the analysis of other drugs of abuse.

Introduction

Hair analysis is a powerful tool in forensic and clinical toxicology since many chemical compounds, such as drugs, toxicants and doping substances, are incorporated into hair after intake.[1] The increasing sensitivity of analytical techniques has enabled the detection of very low concentrations in hair and caused an increasing use of hair analysis in drugs of abuse screening and in clinical applications. Hair analysis has several advantages over more traditional biological matrices such as urine and blood; sample collection is easy and non-invasive and the detectability of compounds upon intake can be prolonged to months or even years instead of days.[2,3] Analysis of longitudinal segments allows the assessment of retrospective timelines, based on an average hair growth rate of 1.3 cm per month,[4] which is extremely relevant in forensic investigations following ingestion of a single dose. Over the past few decades, many hair analysis methods have been published. Most of them involve digestion of hair (or 1–3 cm long hair segments) followed by the extraction of compounds of interest.[2,5] After further sample clean-up, the extracts are usually analyzed using gas or liquid chromatography (GC or LC) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) providing sensitivity of the order of (sub-)ng per mg for often tested drugs such as cocaine and Δ^9-tetrahydrocannabinol (THC).[6] These conventional segmented hair analysis methods are rather laborious, time-consuming and the 1–3 cm segments provide only a rough estimate of the time of drug intake.

More recently, matrix-assisted laser desorption/ionization (MALDI)-MS has been used to detect drugs of abuse in hairs.[7-12] Extracts from, segmented, pulverized hairs were screened for cocaine by Vogliardi *et al*., resulting in a limit of detection of 0.1 ng/mg hair.[11,12] Miki *et al*. demonstrated the detection of amphetamine by analyzing a longitudinal cross-section of a single hair using MALDI.[7,8] Porta *et al*.[10] used a triple quadrupole linear ion trap to perform MALDI imaging MS experiments on single hairs of chronic cocaine users and were able to detect cocaine concentrations down to 5 ng per mg hair. By longitudinal scanning of a single hair, the authors could obtain detailed historical information concerning drug intake over several months. The very high longitudinal spatial resolution of that approach (~1 mm) could potentially provide an estimate of the time of drug intake in days, versus a month when using conventional segmented hair analysis methods.[10] Although these results represent major scientific achievements, the analysis of single hairs can easily lead to misinterpretation. The growth cycles of individual hairs are not synchronous: 80–90% is in the anagen phase and growing, while the remainder of the hairs is in the regressing catagen or resting telogen phase and not growing.[1,13] Hence, single hair analysis can cause false-negatives and incorrect conclusions with regard to the retrospective timeline assessment due to these differences in growth cycles. This has been observed in practice by Musshoff *et al*.[9] while analyzing four single hairs of a drug abuser with MALDI imaging MS: using a sophisticated orbitrap MS instrument they were able to detect cocaine, its metabolites and cannabinoids down to sub-ng per mg levels in two single hairs, but the other two hairs did not show any drugs at all. Averaging the results of several hairs from one individual followed by MALDI hair scanning could overcome this problem, but to the best of our knowledge, this has not been demonstrated.

As an alternative, we propose an ambient ionization MS hair scan approach targeting entire locks of hair without the addition of MALDI matrix or any other sample preparation. Those conditions can be met by Direct Analysis in Real Time (DART)-MS. DART was introduced in 2005 by Cody *et al*.[14] as a new ionization technique for fast and direct analysis under ambient conditions. A glow discharge inside the DART source is used to yield metastable species of a heated gas (e.g. helium), which desorb analyte ions from a sample surface at elevated temperatures.[14] DART-MS has shown its ability to screen for several drugs of abuse in different matrices without any sample preparation within minutes.[15] In recent publications, the capability of DART-MS to analyze THC on cotton-swab wipes[16] and synthetic cannabinoids in herbal samples has been demonstrated.[17,18]

In this study, THC has been used as a model compound for the development of a novel DART-orbitrap MS hair scan method. THC is the psychoactive constituent of cannabis and is recommended by the Society of Hair Testing (SoHT) as an indicator of chronic cannabis use with a cut-off value of 0.05 ng per mg hair.[5]

Experimental

Chemicals and materials. Quinine and methanol (HPLC gradient grade) were purchased from J.T. Baker Chemicals (Deventer, The Netherlands). Sodium hydroxide, formic acid and acetic acid were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Acetonitrile, *n*-hexane and ethyl acetate were purchased from Actu-All Chemicals (Oss, The Netherlands) and were of Ultra LC–MS (acetonitrile) or HPLC (*n*-hexane and ethyl acetate) grade. Dichloromethane was purchased from BioSolve (Valkenswaard, The Netherlands). The THC and

THC-d_3 standards were from Lipomed (Arlesheim, Switzerland) and Cerilliant Corporations (Round Rock, TX, USA), respectively.

Secondary THC standard from cannabis extract. Cannabis (Cannabis sativa) leaves and tops were crushed using a mortar. Of the crushed material 4.6 g were extracted for 30 min using 250 mL of methanol under continuous stirring. The extract was filtered over a filter paper (Schleicher & Schuell, Dassel, Germany: No. 595; diameter 70 mm) and stored in a glass bottle at 7 °C. According to LC–MS/MS analysis, this stock solution contained 339 ppm Δ^9-THC.

Dilutions of secondary THC standard solution. The secondary THC standard solution was diluted with methanol to yield different concentrations, ranging from 0.11 to 85 ppm. Quinine, dissolved in methanol/formic acid (99:1 v/v), was added at a constant concentration (25 ppm) both as an internal standard and to visualize hair spiking by fluorescence under UV-light (366 nm).

LC–MS/MS reference method. An Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Micromass Quattro Ultima Platinum (Waters) mass spectrometer was used for LC–MS/MS analysis. Ionization was achieved by positive electrospray ionization using the following conditions: capillary voltage, 3.0 kV; cone voltage, 35 V; source temperature, 120 °C; desolvation temperature, 450 °C; nitrogen cone gas flow rate, 150 L/h; nitrogen desolvation gas flow rate, 600 L/h; collision energy, 30 eV. The following multiple reaction monitoring (MRM) transitions were used: *m/z* 315.3 ($[M+H]^+$) to *m/z* 193.2 ($[M+H–C_9H_{14}]^+$) and 259.3 ($[M+H–C_4H_8]^+$) for THC, and *m/z* 318.3 ($[M+H]^+$) to *m/z* 196.2 ($[M+H–C_9H_{14}]^+$) and 262.3 ($[M+H–C_4H_8]^+$) for THC-d_3. Data analysis was performed using MassLynx V4.1 (Waters). A Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA, USA), thermostated at 50 °C, was used for the separation. The mobile phases were 5 mM formic acid in water (mobile phase A) and acetonitrile (mobile phase B) and the flow rate was 0.8 mL/min. After 2 min isocratic elution with 25% B, a gradient was applied towards 100% B in 7 min, and after 2 min at this condition the system was re-equilibrated for 3 min at 25% B. Initial in-house validation of the method showed good reproducibility (relative standard deviation (RSD) <10%). The recovery of THC in spiked hair at three different levels (0.04, 0.2 and 2 ng/mg) and without internal standard correction was found to be 55% (RSD 21%). Based on these data the reference method was considered fit for purpose. THC levels in hair were determined using a matrix-matched calibration curve (R^2 = 0.99) prepared by spiking of THC standard to blank hair and internal standard correction.

DART-orbitrap MS analysis. Analysis of spiked and drug-user hair samples was performed using a DART-SVP ion source (IonSense, Saugus, MA, USA) coupled to an Exactive orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Thermo Scientific Xcalibur software (V2.1.0.1139) was used for data acquisition and processing. The measurements were performed using a scan range of *m/z* 100.0–2000.0, a mass resolution of 100,000 (full width at half maximum, FWHM) at a scan rate of 1 Hz and a maximum inject time of 100 ms. The DART ambient ionization source was used in combination with the X-Z transmission module (IonSense) and operated in positive ion and transmission mode. Helium was used as the ionizing gas at a flow rate of ~3.7 L/min. Hair samples, corresponding to approximately 150–200 hairs and a weight of approximately 10 mg per cm, were attached to a stainless steel mesh screen (opening size 0.24 × 0.24 mm) using adhesive tape and placed inside the X-Z module holder, which had slits of 113 mm width × 7 mm height (see Figure 1A). The DART source was positioned 5 mm from the sample, which was 3 mm from the ceramic inlet tube of the mass spectrometer (Figure 1A). The hair samples were longitudinally scanned in the X-direction at a scan speed of 0.2 mm/s.

A few additional experiments were performed on a Q-Exactive orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific) in order to assess the increased sensitivity of more recent instruments towards DART hair scanning of THC. The measurements were performed at a mass resolution of 140,000 (FWHM), a scan rate of 0.5 Hz and a maximum inject time of 200 ms. The instrument was operated in MS/MS mode with precursor ion selection on the $[M+H]^+$ ion (*m/z* 315.0± 1.0), a normalized collision energy setting of 10.0 eV and a product ion scan range of *m/z* 50.0–350.0. In short, blank hair samples were spiked using more diluted secondary THC standard solutions as described below and the resulting THC signal was compared with that obtained using an Exactive mass spectrometer.

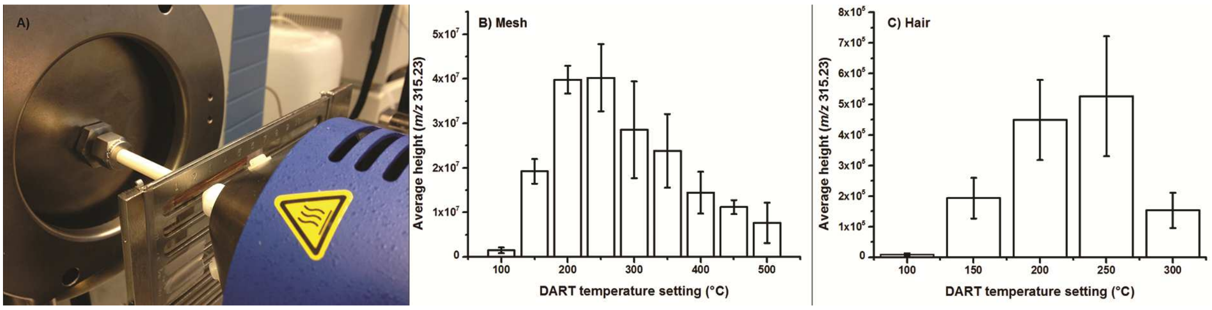

Figure 1. *A) Experimental setup used for hair analysis by DART-orbitrap MS using the X-Z transmission module. Average DART-orbitrap MS signal intensity for THC (m/z 315.2319) spiked on B) stainless steel mesh sheet, and C) blank hair at different DART helium temperature settings.*

Decontamination of hair. The decontamination procedure was adopted from Salomone *et al.*[19]: hair samples were decontaminated using two 3 mL dichloromethane washes for 3 min while vortexing. After being dried at room temperature, the decontaminated hair samples were used for DART-MS measurement or further pretreated as described below for LC–MS/MS analysis.

LC–MS/MS sample preparation. The sample preparation for LC–MS/MS analysis was adopted from Salomone *et al.*[19] with slight modifications. In short, 30–50 mg of decontaminated hair sample were cut into small pieces (<3 mm) and 5.0 µL of 1 ppm THC-d_3 in methanol was added. The hairs were then incubated in 1.0 mL NaOH for 10 min at 95 °C and, after cooling to room temperature, the pH was adjusted to 2 using concentrated acetic acid. Liquid-liquid extraction with 3.0 mL of *n*-hexane/ethyl acetate (90:10) was performed. The organic phase was separated and dried under a gentle N2 flow at 40 °C. The dried sample was reconstituted in 100.0 µL of methanol and a 5.0 µL aliquot was injected into the LC–MS/MS system.

Hair samples. For spiking of hair with the secondary THC standard, blank hair samples were collected from non-cannabis users. Positive hair samples were collected anonymously from eleven individual chronic cannabis users using the recommended forensic hair collection protocol[5] in the cities of Wageningen and Maastricht (The Netherlands). The users stated that they had used cannabis at least thrice a week during the previous 6 months.

Spiking of blank hair samples. Diluted secondary THC standard solutions were very briefly (<1 s) sprayed onto blank hair samples using a thin layer chromatography (TLC) nebulizer from a distance of 10 cm. Prior to the spraying, a 1 mm thick stainless steel mask with different slits (1 × 10, 2 × 10 or 5 × 10 mm) was placed on top of the hair samples to expose only a small longitudinal section of hair to the spray. This approach enabled accurate spiking of small zones onto locks of blank hair, which could be confirmed both by the blue-white fluorescence of quinine at 366 nm and by DART-orbitrap MS measurements.

Determination of optimum DART helium gas temperature. The optimum DART gas temperature was investigated using stainless steel mesh screens and blank hair by spiking with 2.0 µL of the secondary THC standard solution. The spiked zones were then analyzed using the DART-orbitrap MS method described above while adjusting the DART gas temperature setting from 100 to 500 °C at 50 °C intervals.

Determination of the DART spot size in the hair scan. To determine the spot size probed by the DART ionization beam, blank locks of hair were spiked using the described spraying technique through a 2 × 10 mm slit. This resulted in a 2 mm longitudinal section of the hair spiked with THC, and this was visually confirmed using the fluorescence of quinine at 366 nm. After DART-orbitrap MS measurement, the length of the zone in seconds was determined in the extracted ion chronogram (EIC) of the THC ($[M+H]^+$ ion; *m/z* 315.2319) and converted into scanned millimeters (scan rate was 0.2 mm/s). The length of the actual spiked zone (2 mm) was then subtracted and the result was divided by 2 to obtain the one-sided deviation of the zone in mm (Supplementary Figure S2, Supporting Information). To investigate a potential decrease of the DART spot size, different stainless steel masks with a small slit were placed between the DART source and the hair sample. Slits down to 0.35 mm were used and the one-sided deviation of the zone in mm was determined as described above.

Sensitivity and linearity experiments. For the sensitivity and linearity experiments, dilutions of secondary THC standard solution were prepared as described. The sensitivity and linearity of the method were investigated on mesh by pipetting 5.0 µL of the dilutions over approximately 30 mm2 of mesh (5 × 6 mm). The previously described TLC spraying method was used to spike locks of blank hair using serial dilutions of secondary THC standard solution. For the calibration curves, the ratio between the MS signals of THC ($[M+H]^+$; *m/z* 315.2319) and quinine ($[M+H]^+$; *m/z* 325.1916) was used as the relative response.

Results and Discussion

DART-orbitrap MS conditions

Since THC was used as model compound in this research, the orbitrap mass spectrometer was tuned on the corresponding $[M+H]^+$ signal (*m/z* 315.2) using direct infusion of the cannabis extract with electrospray ionization (ESI). A full spectrum of the secondary THC standard solution, with THC present as $[M+H]^+$ (*m/z* 315.2324), is shown in Supplementary Figure S1 (Supporting Information). The DART conditions were optimized by measuring 2 µL spots spiked on a stainless steel mesh screen with different DART helium gas temperatures. Mesh temperatures of 200 and 250 °C resulted in the highest signals for *m/z* 315.2319 (Figure 1B). At lower temperatures THC is probably not desorbed from the mesh, while at higher temperatures THC probably decomposes, resulting in lower intensities. The same experiment was conducted on spiked blank hair. This resulted in an optimum setting comparable with that on mesh at 200 and 250 °C (Figure 1C). Note that temperature settings above 300 °C were not considered for hair, since hair starts to singe beyond this setting. Based on these results, the DART source was set to 250 °C in all further experiments. However, the actual temperature near the hairs is much lower and approximately 135 °C, as evidenced by thermocouple measurements.

Performance of the DART-orbitrap MS scanning using spiked hair samples

To investigate the performance of the hair scan concept, spiked hair samples were used. Therefore, blank hair samples were spiked with secondary THC standard through a mask with narrow slits (1 × 10 or 2 × 10 mm) using a TLC nebulizer. Quinine was added to the secondary THC standard for visual confirmation of the spiking. This technique allowed spiking of narrow zones of 1 or 2 mm length on a lock of blank hair, which was impossible to achieve by a syringe or pipette due to capillary action of the lock of hair. The samples were scanned in the longitudinal direction from scalp to the tip end using the X-Z transmission module of the DART source at a scan speed of 0.2 mm/s. Figure 2 shows both a visual and a mass spectrometric analysis of a narrow zone of THC spiked onto a lock of hair.

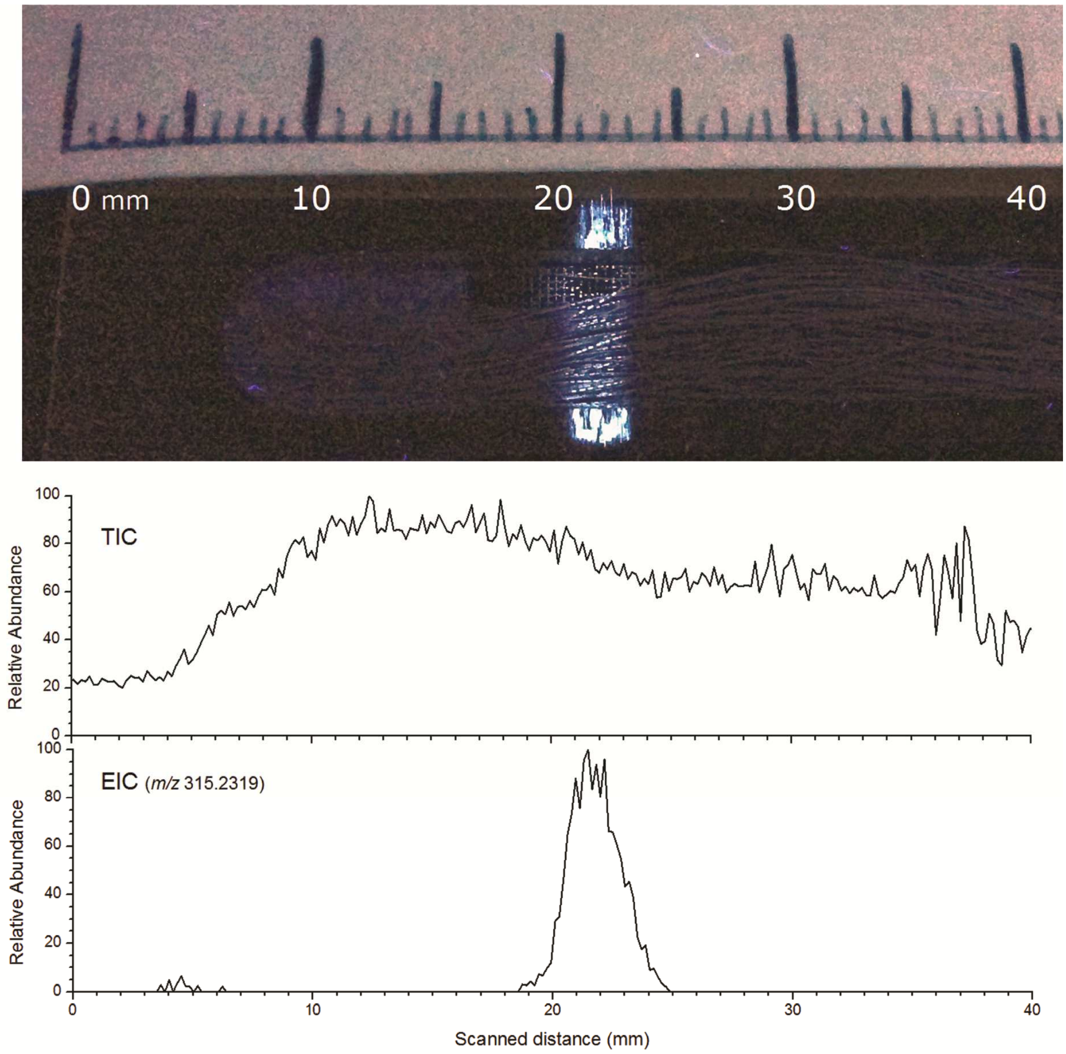

Figure 2. *DART-orbitrap MS hair scan after spiking of a lock of blank hair with secondary THC standard and quinine using a TLC nebulizer. Top: fluorescence of quinine at 366 nm; middle: total ion current (TIC); bottom: extracted ion chronogram (EIC) of THC.*

The repeatability and reproducibility of the spiking and DART-orbitrap MS hair scanning were tested following spiking of three longitudinal zones on a lock of blank hair using the described method (repeated eight times on different locks of blank hair). The average one-sided zone deviation was determined as described in the Experimental section and Supplementary Figure S2 (Supporting Information). The spiking technique and DART hair scan method were shown to be very reproducible with regard to the average one-sided deviation of the zone measured (RSD <20%, Table 1). The measured average intensity of the THC signal, corrected using quinine as internal standard, showed RSD values (Table 1) in accordance with typical quantitative DART performance as observed in other studies.[20-22] Overall, it can be stated that the repeatability and reproducibility of measurements of spiked locks of blank hair using a TLC nebulizer and the new developed DART-orbitrap MS hair scan method are sufficient for the purpose of a rapid pre-screening method for THC in hair.

Table 1. *Repeatability and reproducibility of measurements of spiked locks of blank hair using the DART-orbitrap MS hair scan method. Relative intensity of $[M+H]^+$ ion of THC (m/z 315.2319) versus that of quinine (m/z 325.1911). For explanation, see text and Supplementary Figure S2 (Supporting Information)*

	Average one-sided zone deviation (mm)	RSD	Relative average intensity	RSD
Repeatability (n=3)	2.49	19%	0.17	26%
Reproducibility (n=8)	2.52	16%	0.14	27%

The spot size of the DART beam on hair can be derived from the data in Table 1. The average deviation of the longitudinal zone measured and the actual zone spiked (visually confirmed under UV light) indicate the spot size probed by the DART ionization beam. This deviation was approximately 2.5 mm on both sides of the zone, indicating a spot size of approximately 5 mm, which corresponds to the typical DART beam dimensions reported by Chernetsova *et al*.[23] Therefore, the newly developed DART hair scan method would provide a retrospective timeline accuracy of ±2 weeks, assuming an average hair growth rate of 1.3 cm per month.[4] This is potentially a significant and forensically relevant improvement over conventional hair analysis techniques, in which 1–3 cm hair segments are used (corresponding to a 1–3 month timeline) but not as accurate as the ~1 mm spatial resolution obtained in single hair MALDI imaging. In an attempt to decrease the spot size of the DART beam different stainless steel masks (having slit widths of 0.35, 0.5, 0.75 and 1 mm) were developed and positioned in front of the hair sample. Unfortunately, this did not yield narrower signals in the EIC, probably due to turbulence caused by the hair sample.

Several secondary THC standard dilutions (with constant quinine concentration) were spiked and measured both on mesh and on blank hair to determine the sensitivity and linearity of the DART hair scan method. 5.0 µL of 4–400 times diluted secondary THC standard solutions, corresponding to THC concentrations of 0.85 to 85 ppm, were spiked on mesh (absolute amounts spiked ranging from 4.25 to 425 ng) resulting in a linear calibration curve with an R^2 value of 0.998 for the average intensity of the THC signal. The relative response versus the MS signal of quinine ($[M+H]^+$; *m/z* 325.1916) as internal standard showed a similar R^2 value of 0.992 (Figure 3B vs 3A). Next, dilutions of secondary THC standard solution from 10 - 100 times, corresponding to 34 to 3.4 ppm THC, were sprayed on blank hair through a slit of 2 mm width.

The resulting calibration curve had a poor R^2 value of 0.881 for the average intensity of the THC signal, but when using quinine as internal standard, the linearity improved significantly to an R^2 value of 0.991 (Figure 3D versus 3C). Obviously, an internal standard is necessary for correcting differences in spiked THC levels on hair due to variations in the application using a TLC sprayer (see also Table 1).

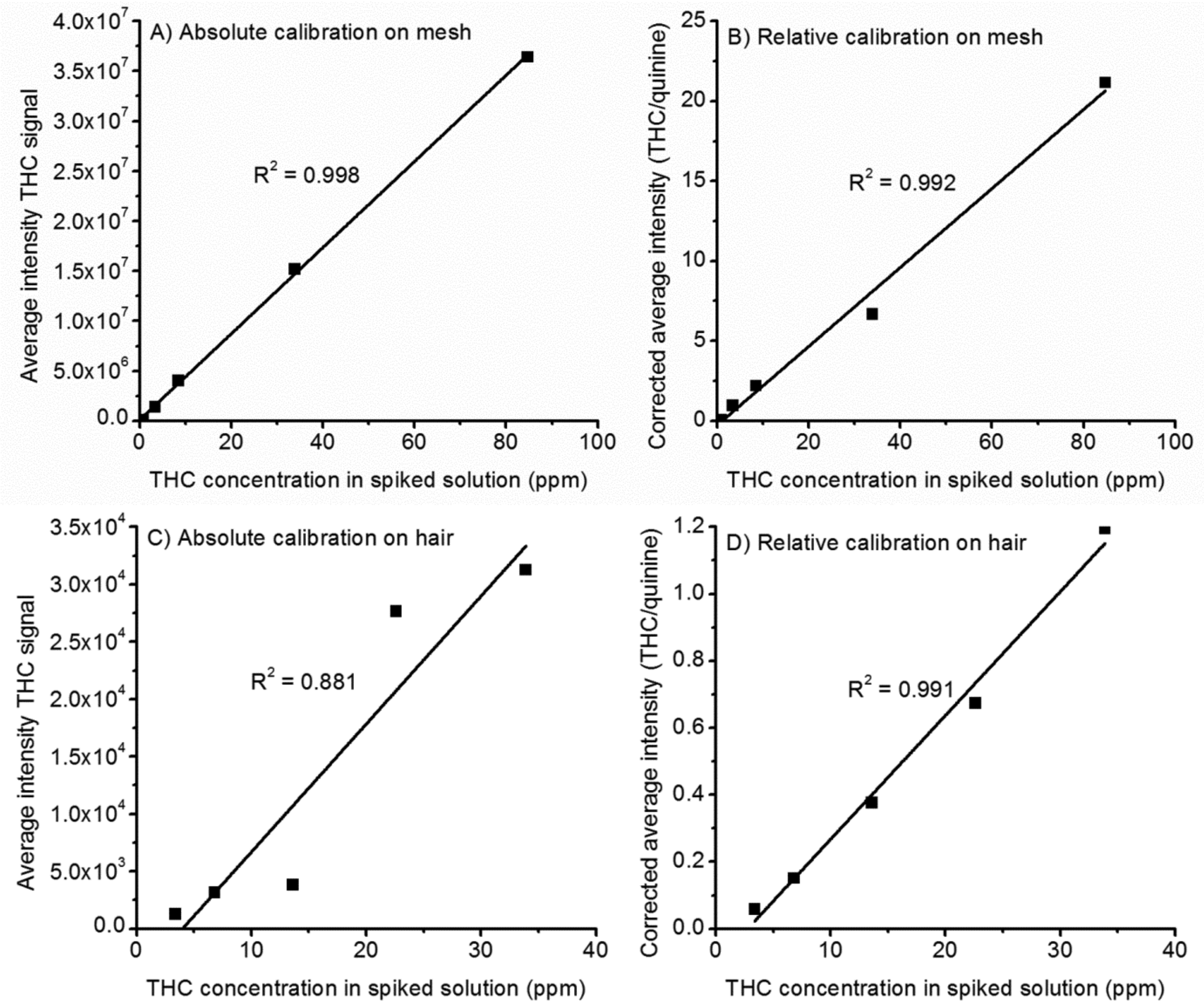

Figure 3. *Absolute and relative calibration curves for dilutions of secondary THC standard solution measured on mesh and hair.*

With the TLC spraying technique it is not possible to apply an exactly known amount of THC to a lock of hair. Therefore, spiked locks of hair were also analyzed using the quantitative LC–MS/MS reference method. Spiking by means of spraying of a 10 times diluted secondary THC standard solution through a 2 mm slit yielded an average amount of 46 (± 14) ng THC per mg hair. The detection limit of the DART hair scan using a benchtop orbitrap mass spectrometer was reached when spraying a 100 times diluted secondary THC standard solution, which would correspond to roughly 5 ng THC per mg hair. Consequently, with the present DART hair scan MS system using a relatively simple benchtop orbitrap instrument, THC can be detected in hair samples from chronic users with detection limits comparable with those achieved for cocaine by Porta *et al*.[10] using MALDI imaging MS. However, to prove single dose THC intake, the sensitivity of the method should be further improved in the future. Compared with preliminary MALDI imaging measurements performed by Musshoff *et al*.[9] using a more advanced orbitrap instrument, the current DART hair scan method is somewhat less sensitive but much more simple to perform thanks to the ambient ionization of THC from entire locks of hair. Related to the cut-off value for THC of 0.05 ng/mg recommended by the SoHT,[5] the DART hair scan method on a benchtop orbitrap is not sensitive enough yet for official forensic hair testing. However, preliminary experiments using a more sensitive Q-Exactive orbitrap mass spectrometer showed a large increase in sensitivity, yielding a detection limit for THC in hair of 0.3 ng/mg. In other words, the DART approach has already come close to the official THC requirement. Note that the SoHT-recommended THC cut-off value is much lower than that for cocaine[5] so the latter is probably already analyzable by DART-orbitrap MS (cocaine could not be tested yet in our setup due to legal restrictions).

Performance of the DART-orbitrap MS hair scan method on samples from cannabis users

Eleven hair samples were tested and THC was successfully detected in six of the eleven samples using the developed DART hair scan method. Note that another cannabinoid that can be found in cannabis, cannabidiol (CBD), has the same elemental composition and thus the same theoretical exact mass. So in real hair samples the EIC actually represents THC and/or CBD. However, according to LC–MS/MS analysis, the THC levels were much higher than the CBD levels in these samples. The THC content in the positive samples was also quantitatively determined using LC–MS/MS and it was found to be 5.8 to 8.7 ng THC per mg hair. According to literature reports,[19,24-26] these levels are relatively high but not unexpected given the fact that cannabis in The Netherlands is known for its exceptionally high THC levels. This confirms the estimated detection capability of the DART hair scan method, being able to screen for THC in hairs of chronic heavy cannabis users (levels >1 ng/mg). Nevertheless, it is fair to say that the SoHT cut-off value for chronic users is 20× lower; thus, for compliance with the THC cut-off a more sensitive mass spectrometer such as a quadrupole orbitrap or a triple quadrupole should be employed. Figure 4 shows the TIC and EIC of *m/z* 315.2319 from a DART hair scan of a specific individual cannabis user hair sample (5.8 ng THC per mg hair according to LC–MS/MS). The first and last 2.5 mm of the lock of hair, both corresponding to approximately 1 week of hair growth, were used to attach the sample onto the stainless steel mesh and could therefore not be analyzed. The resulting hair scan distinguishes three sections from the root to the tip end of the hair; a first section, from 2.5 to 7 mm, in which much THC was detected, a second section, from 7 to 14.5 mm, where less THC was detected, and a third and last section, from 14.5 to 23.5 mm, where almost no THC was detected. These sections correspond to, respectively, 1.5, 2.5 and 3 weeks of hair growth history, indicating that the sample contained more THC at the scalp side than at the tip end of the hairs. Possibly, this specific individual used cannabis at a higher frequency in recent weeks. Other hair samples showed different patterns (see e.g. Figure 5A). Irrespective of the cause, the result of Figure 4 shows that the DART hair scan can distinguish changing THC levels in different longitudinal sections of a hair sample and would be potentially applicable to retrospective timeline assessment of incidental drug intake. Since the possibility of external contamination is a major issue in forensic hair analysis, hair samples were measured before and after dichloromethane decontamination to be able to confirm the detection of incorporated THC versus external contamination. Two cannabis user samples with high THC content were used for this test. The sampled locks of hair were divided into four sub-samples, which were analyzed before or after decontamination using the developed DART hair scan method or the LC–MS/MS reference method. Based on the LC–MS/MS results, the total THC content was reduced by only 12% by the dichloromethane decontamination procedure. This strongly suggests that the major part of the detected THC content is incorporated THC. The next step was to confirm the ability of the DART hair scan method to detect this incorporated THC. The untreated sub-sample and the decontaminated sub-sample were analyzed and THC was found in both sub-samples: Figure 5 shows the DART hair scans of an untreated (Figure 5A) and decontaminated sub-sample (Figure 5B). Small differences can be noticed between these figures, indicating that some THC was removed during decontamination. To prove the validity of the applied decontamination procedure, blank hair samples were spiked (i.e. intentionally externally contaminated by spiking) and measured with and without the decontamination step (Figures 5C and 5D). The resulting hair scans clearly showed high THC signals without decontamination, but no THC was detected at all after decontamination of the externally contaminated hairs. The successful detection of THC in decontaminated drug user hair samples convincingly demonstrates that the DART hair scan not only probes THC at the hair surface, but penetrates deeply enough to measure incorporated THC. Note that according to SoHT guidelines[5] cannabis use can only be fully proven when both THC and its main metabolite THC-COOH are detected.

However, the latter is present in hair only in very low concentrations (recommended cut-off = 0.0002 ng/mg[5]) and therefore not detectable using a DART hair scan. So the DART hair scan should be considered as a rapid pre-screening method, followed by highly sensitive GC– or LC–MS(/MS) analysis for THC-related metabolites. The preliminary results of repeated longitudinal hair scans show a difference in the rate of decrease of the measured THC signal between spiked ('contaminated') blank hair samples and real drug user samples (Figure 5E). These results suggest that the DART hair scan could pinpoint external surface contamination via repeated scanning, without the analysis of wash solutions.

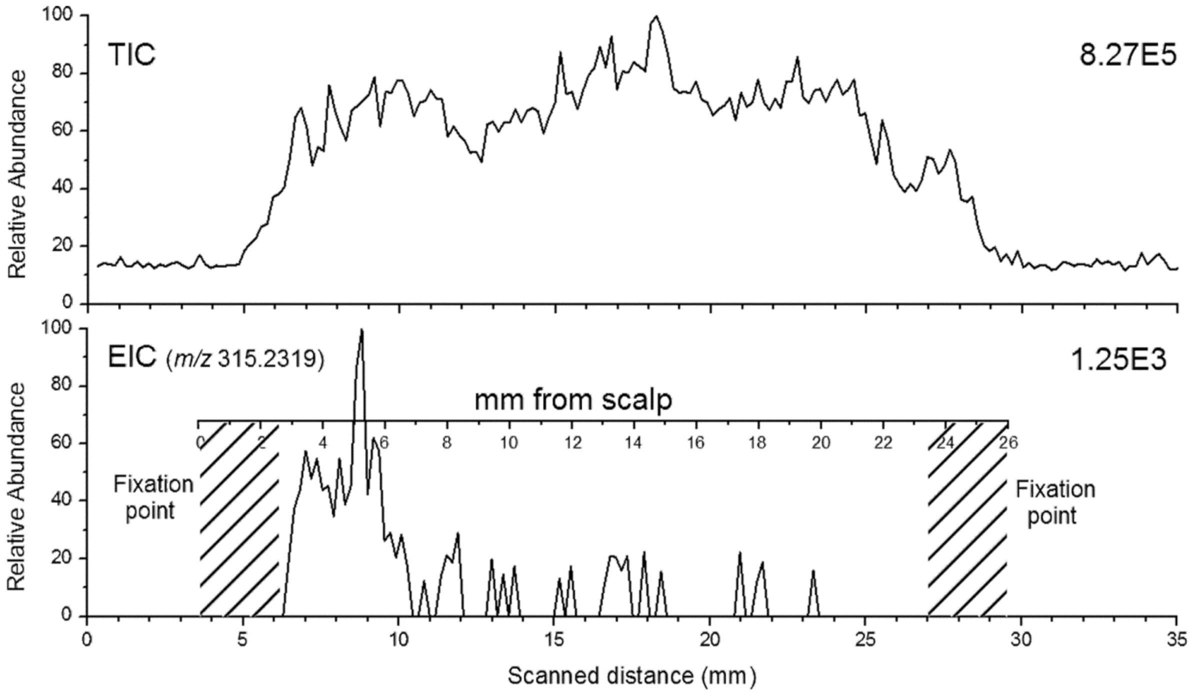

Figure 4. *TIC and EIC of m/z 315.2319 from a DART hair scan (scan rate = 0.2 mm/s) of a cannabis user hair sample.*

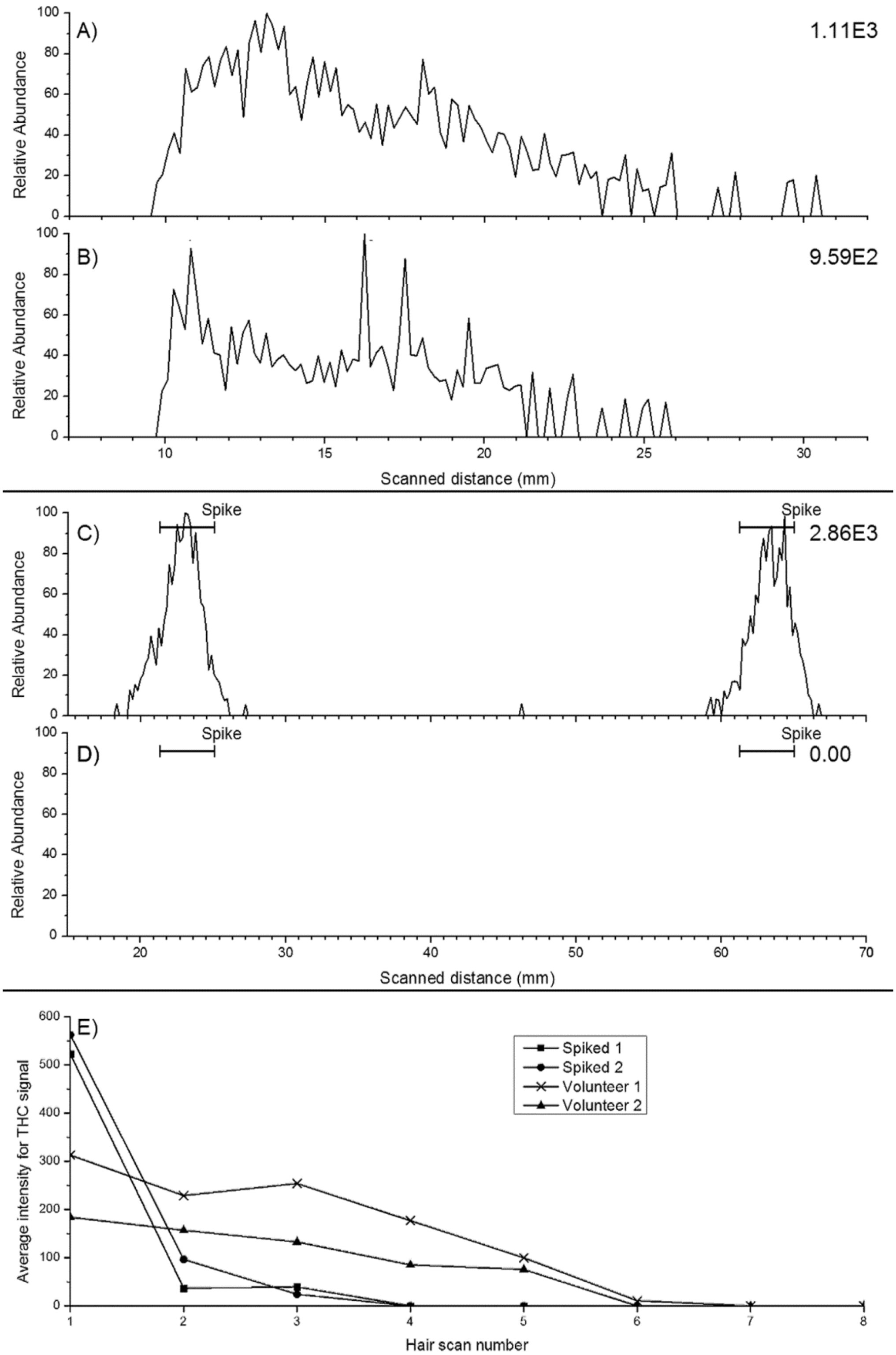

Figure 5. *EIC of m/z 315.2319 of DART hair scans from A) drug user hair subsample, B) a decontaminated drug user hair sub-sample, C) a spiked ('contaminated') blank hair sample, and D) a decontaminated spiked blank hair sample. E) Average intensity of THC signal in repeated longitudinal hair scans of spiked blank hair samples and drug user samples.*

Conclusions

In this paper, a new approach for forensic hair analysis is presented. Instead of using digestion of hair or digestion of multiple 1–3 cm hair segments followed by conventional LC– or GC–MS analysis, complete locks of hair were probed under ambient conditions using DART. Thanks to the lack of sample preparation a fast pre-screening for drugs of abuse, such as THC, is possible. By using entire locks of hair the analysis is not hampered by differences in growth cycles between individual hairs. As a proof of principle, THC was detected in hairs of chronic cannabis users using a benchtop orbitrap instrument. Since several other drugs of abuse have already been analyzed in other sample matrices using DART-MS,[16] it is envisaged that the hair scan method could be expanded to other compounds like cocaine, heroin, amphetamines and tranquillizers, even including unexpected designer drugs and their metabolites thanks to the full scan accurate mass data that allow retrospective interrogation. Initial tests showed that more advanced orbitrap MS systems are capable of pushing the DART hair scan detection limit into the sub-ng per mg range, which is highly relevant for single dose drug administrations and retrospective timeline assessments, such as in drug-facilitated crime cases. Of course, the presented THC screening method is less relevant for performing retrospective timeline assessment and single dose drug administration but it shows the potential of the DART hair scan method. For drug-facilitated crime cases, the sensitivity could be increased towards the pg per mg range using a triple quadrupole mass spectrometer. Next the hair scan method presented should be validated as a pre-screening tool using different hair type samples from multiple sources, including pigmented and damaged hair, in order to be eventually adopted by the forensic hair analysis community.

Acknowledgments

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Supporting information Chapter 3

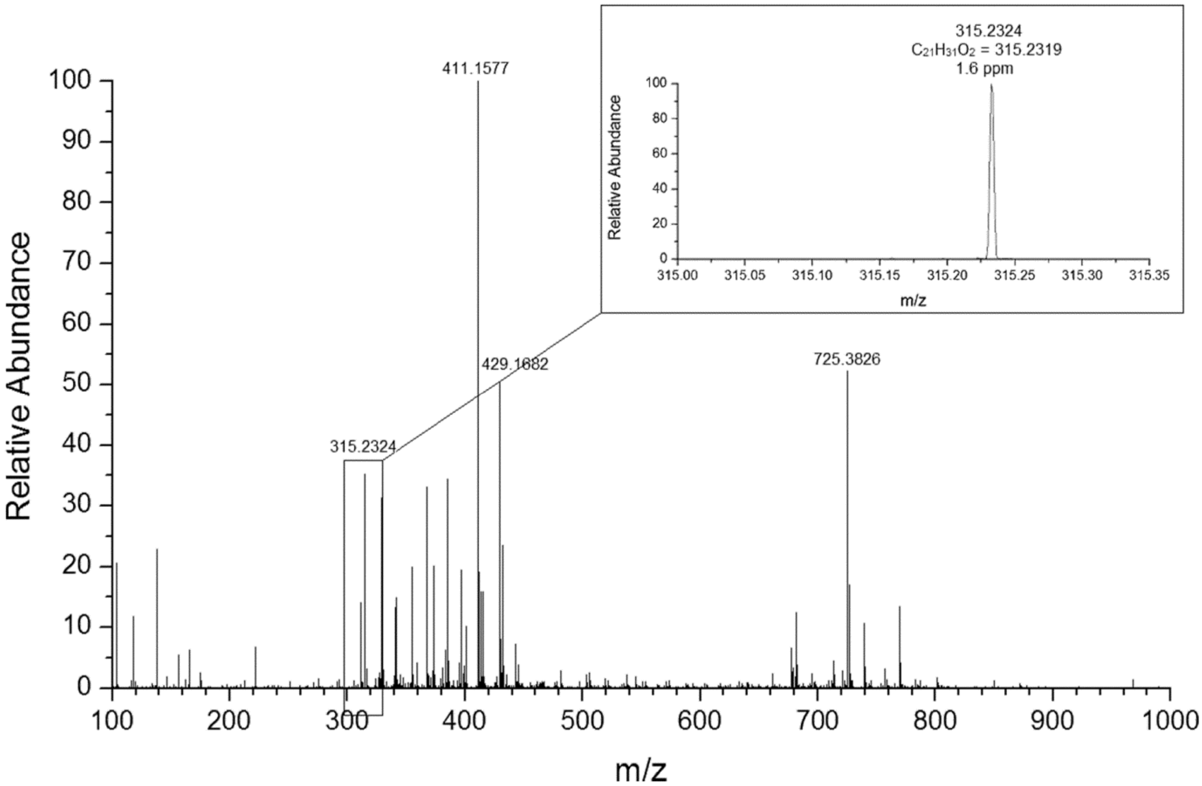


Figure S1. *Full positive ESI-orbitrap MS scan of direct infusion of cannabis extract used as secondary standard in spiking experiments showing the presence of THC ([M+H]$^+$; m/z 315.2324).*

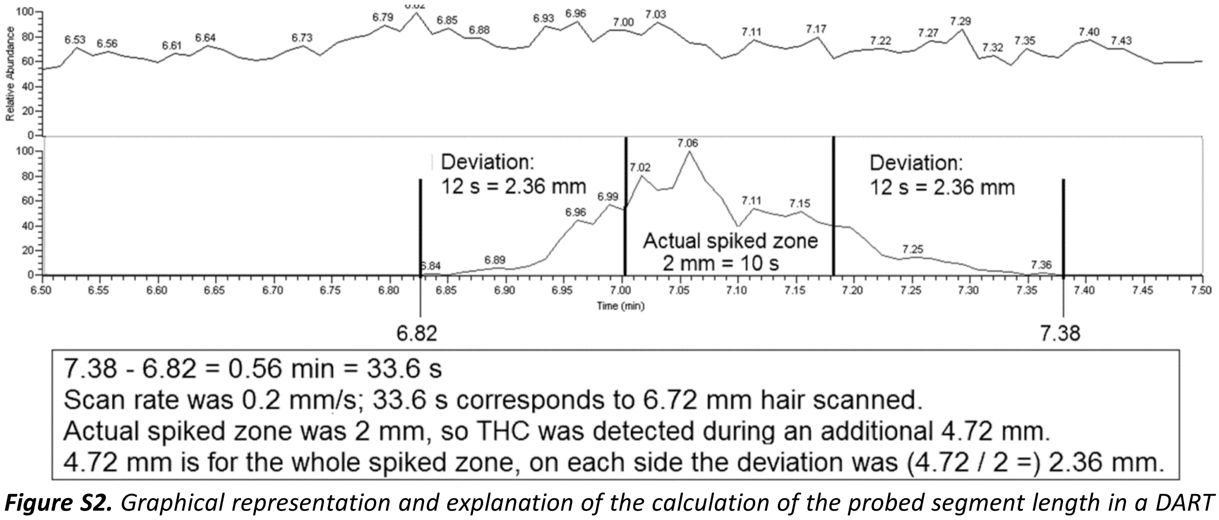
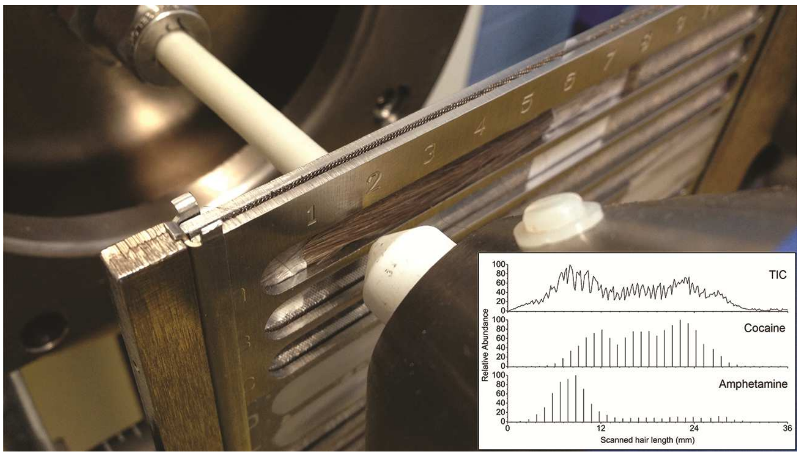


Figure S2. *Graphical representation and explanation of the calculation of the probed segment length in a DART hair scan and the deviation from the zone actually spiked.*

Chapter 4

(Un)targeted Scanning of Locks of Hair for Drugs of Abuse by Direct Analysis in Real Time − High-Resolution Mass Spectrometry



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Abstract

Forensic hair evidence can be used to obtain retrospective timelines of drug use by analysis of hair segments. However, this is a laborious and time-consuming process, and mass spectrometric (MS) imaging techniques, which show great potential for single-hair targeted analysis, are less useful due to differences in hair growth rate between individual hairs. As an alternative, a fast untargeted analysis method was developed that uses direct analysis in real time−high-resolution mass spectrometry (DART-HRMS) to longitudinally scan intact locks of hair without extensive sample preparation or segmentation. The hair scan method was validated for cocaine against an accredited liquid chromatography/tandem mass spectrometry (LC–MS/MS) method. The detection limit for cocaine in hair was found to comply with the cut-off value of 0.5 ng/mg recommended by the Society of Hair Testing; that is, the DART hair scan method is amenable to forensic cases. Under DART conditions, no significant thermal degradation of cocaine occurred. The standard DART spot size of 5.1 ±1.1 mm could be improved to 3.3 ±1.0 mm, corresponding to approximately 10 days of hair growth, by using a high spatial resolution exit cone. By use of data-dependent product ion scans, multiple drugs of abuse could be detected in a single drug user hair scan with confirmation of identity by both exact mass and MS/HRMS fragmentation patterns. Furthermore, full-scan high-resolution data were retrospectively interrogated versus a list of more than 100 compounds and revealed additional hits and temporal profiles in good correlation with reported drug use.

Introduction

Many compounds, including a large range of drugs of abuse, are incorporated into the hair matrix by passive diffusion from blood in the hair follicle.[1,2] Over the past decade, hair analysis for drugs of abuse has developed from methods using traditional techniques like liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS),[3,4] via the application of new ionization techniques like matrix-assisted laser desorption/ionization (MALDI) on pulverized hair[5,6] toward state-of-the-art imaging methods using MALDI, laser ablation electrospray ionization (LAESI), or metal assisted-secondary ion mass spectrometry (MetA-SIMS) instruments.[7-19] Where LC–MS/MS and GC–MS/MS are still the techniques of choice for routine quantitative measurements, scanning of hair by MS imaging techniques can be used to obtain a continuous retrospective timeline of drug intake. Previously, this could only be achieved by segmenting hair samples prior to analysis, a laborious and time-consuming process. Segments consisting of 1−3 cm of hair are generally used,[3,20,21] representing multiple months of hair growth (typical hair growth rate is around 1.0 cm/month),[10,21-26] while spatial resolutions down to 30 μm with MALDI,[12] 200 μm with LAESI,[8] and 1 μm with SIMS[9] have been reported. Utilizing these excellent spatial resolutions, even analysis of longitudinal cross sections of a single hair is possible by use of MALDI and SIMS.[9-12,14,18]

MALDI imaging of single hairs can provide a better understanding of drug incorporation mechanisms, drug behavior throughout the hair, and the effect of external contamination.[9,10] However, the routine use of such imaging techniques for forensic cases is limited by several factors: use of a single hair to obtain a retrospective timeline of drug use is doubtful due to differences in growth cycles between individual hairs,[13,18,27-29] but on the other hand, the analysis of multiple single hairs will considerably expand the analysis time. Sample preparation is time-consuming and not trivial due to the necessary application of matrix for MALDI and MetA-SIMS analysis. Moreover, in most studies, single hairs are imaged for targeted analysis of a single drug or a few drugs only, and the total analysis time can be as long as multiple days.

An alternative to single-hair imaging techniques is direct analysis in real time (DART) analysis of intact locks of hair. DART is an ionization technique using heated, metastable gas species (e.g., helium) to desorb ions from a surface under ambient conditions.[30-32] DART has been demonstrated as a useful technique for many different matrices[30,33-41] and has great possibilities for rapid screening of drugs of abuse with minimal sample preparation.[33,42-48] A previous feasibility study showed the potential of DART−high-resolution mass spectrometry (HRMS) to directly scan hair samples for Δ^9-tetrahydrocannabinol (THC), the main active component of cannabis, to obtain a retrospective timeline of cannabis use.[49] Analysis of a lock of hair, consisting of 150–200 hairs, circumvents the problem that arises in single-hair analysis due to different hair growth phases. Hair scans were obtained within minutes, making the method suitable for rapid routine analysis. Although proof-of-principle of DART as a fast screening method targeting intact locks of hair was demonstrated, only THC was investigated. In addition, no confirmation of identity nor metabolite detection could be achieved, and the sensitivity for THC was not in the Society of Hair Testing (SoHT) recommended cut-off range.

In this work, we report a new hair scan method for simultaneous targeted and untargeted detection of multiple drugs of abuse and metabolites in hair without extensive sample preparation. Both full-scan and simultaneous data-dependent product ion scans for additional confirmation of the identity of the detected compounds, and improvement of the DART spot size by use of a smaller DART exit cone, is demonstrated. Moreover, the recorded full-scan high-resolution data were retrospectively interrogated versus an extensive list of drugs of abuse and related compounds, thus revealing individual temporal profiles of both targeted and untargeted drugs of abuse.

Experimental Section

Chemicals and materials. Methanol (HPLC gradient grade) and quinine were purchased from J.T. Baker Chemicals (Deventer, The Netherlands). Dichloromethane (HPLC grade) was purchased from Acros Organics (Geel, Belgium). THC standard was from Lipomed (Arlesheim, Switzerland). Amphetamine, cocaine, and 3,4-methylenedioxymethamphetamine (MDMA) standards were from Cerilliant Corp. (Round Rock, TX, USA).

Secondary cocaine standard and dilutions. Due to legal restrictions, not all experiments could be performed with cocaine standard solutions. Instead, a secondary cocaine standard was obtained from coca tea leaves. For this purpose, 0.55 g of coca tea leaves was added to 50 mL of methanol, and the mixture was continuously stirred for 18 h at room temperature in a closed vessel. After removal of the tea leaves by filtration, the extract was stored in the refrigerator for further testing. The cocaine concentration was determined by a UKAS ISO 17025 accredited reference laboratory and found to be 41 ppm. For spike experiments, several dilutions of the secondary cocaine standard have been made in methanol, ranging from 0.04 to 4.1 parts per million (ppm).

Hair samples. Blank hair used in spiking experiments was obtained from non-drug users. Authentic drug user hair samples were collected from 10 anonymous volunteers in the city of Wageningen (The Netherlands) who declared drug use in the 6 months prior to sampling. Their drug use was recorded anonymously, and informed consent was obtained.

Spiking of blank hair samples. To accurately spike blank hair samples with small zones of a certain analyte, a thin-layer chromatography (TLC) nebulizer was used as described before.[49] In short, blank hair samples were covered by a 1 mm thick stainless steel mask with different slits (1 × 10 and 2 × 10 mm) and the appropriate solution was briefly (<1 s) sprayed in the direction of the mask from a distance of approximately 10 cm, resulting in small longitudinal zones of spiked hair.

Decontamination procedure. Several authentic hair samples were decontaminated prior to analysis by a procedure adopted from literature,[50-54] consisting of two 5 mL dichloromethane washes for 5 min under continuous shaking. After decontamination, the hair samples were dried at room temperature for 15 min and stored for further analysis in aluminum foil at room temperature.

Reference analysis for drugs of abuse. LC–MS/MS reference testing for cocaine, benzoylecgonine (BZE), MDMA, and amphetamine in wash solvents used for hair decontamination and hair samples was performed by Cansford Laboratories (Cardiff, Wales, U.K.) under UKAS ISO 17025 accreditation. The uncertainty of measurement was 17% for cocaine, 38% for amphetamine, and 8% for MDMA.

Sample preparation for DART-HRMS analysis. For DART-HRMS hair scan analysis, samples corresponding to approximately 10 mg/cm, approximately 150−200 hairs, were attached to stainless steel mesh screens (opening size 0.24 × 0.24 mm) by use of 2−3 mm adhesive tape on both ends. The mesh screens were placed inside a DART X-Z module holder (IonSense, Saugus, MA, USA), having slits of 113 mm width × 7 mm height. A picture of the setup used is shown in Figure S1.

Optimization of DART settings. The optimum DART temperature setting for cocaine on mesh or hair was determined by, respectively, spiking 2.0 μL of diluted secondary cocaine standard onto stainless steel screens and spiking of blank hair samples using a TLC sprayer as described above. DART temperature settings were tested with 50 °C intervals from 50 to 500 °C. DART-HRMS sensitivity and linearity were tested by spiking of different dilutions of the secondary cocaine standard onto stainless steel mesh and blank hair. To correct for the error in spiking using the TLC nebulizer, quinine was added to the spike solution as internal standard at a constant concentration of 25 ppm.

DART-orbitrap MS settings. A DART-SVP (simplified voltage and pressure) ion source (IonSense, Saugus, MA, USA) was coupled to an Exactive orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). All full-scan measurements were performed with a scan range of *m/z* 100.0–1000.0 in positive mode, a mass resolution of 100,000 (full width at half maximum, FWHM), and a maximum injection time of 100 ms. The DART-SVP source was operated in positive-ion mode and with a temperature setting of 250 °C unless indicated otherwise. All samples were analyzed in transmission mode with an X-Z transmission module (IonSense) at a scan speed of 0.2 mm/s. As ionization gas, helium was used at a flow rate of ~3.7 L/min.

DART-quadrupole orbitrap (tandem) MS settings. For simultaneous full-scan and data-dependent product ion acquisitions, the DART-SVP ion source was coupled to a Q-Exactive quadrupole orbitrap high resolution mass spectrometer (Thermo Fischer Scientific). Settings used were identical to those described for the DART-orbitrap MS measurements, except for the mass resolution of 140,000 (FWHM). Data-dependent product ion measurements were performed with an isolation width of 1.0 *m/z*, an intensity threshold of 1.0×10^3, a loop count of 1, and a dynamic exclusion of 5.0 s. The inclusion list consisted of cocaine (*m/z* 304.1541), MDMA (*m/z* 194.1177), THC (*m/z* 315.2321) and amphetamine (*m/z* 136.1121). Product ion formation was achieved by using a normalized collision energy (NCE) of 35 for cocaine and THC and 30 for MDMA and amphetamine.

Ultrafast untargeted DART screening without longitudinal scanning. For rapid prescreening of hair samples without longitudinal scanning, hair samples were introduced into the DART ionization region with a pair of tweezers. The samples were moved into and out of the ionization region several times within one analysis cycle to facilitate proper data analysis and differentiation between analysis of the hair sample and background noise.

DART spot size with improved exit cone. The DART spot size, using both the standard exit cone and the high-resolution exit cone as provided by IonSense, was determined as described before.[49] The standard exit cone had an internal diameter of 2.5 mm, while the high-resolution exit cone had an internal diameter of 0.5 mm. Blank hair samples spiked with diluted secondary cocaine standard through 1 × 10 and 2 × 10 mm slits were scanned by use of either of the two exit cones. This procedure was repeated three times for each exit cone.

Retrospective data analysis. Retrospective data analysis was performed with Xcalibur 2.2 (Thermo): full-scan high-resolution data files were loaded into Xcalibur, and the calculated *m/z* values of the drugs of abuse and related compounds listed in Table S1 were plotted with a 5.0 ppm mass tolerance to identify positive hits.

Results and Discussion

Optimization of DART-HRMS settings

Prior to analysis of (drug user) hair samples, the DART-HRMS settings were optimized for analysis of cocaine by use of analytical standards as well as a secondary standard obtained by methanolic extraction of coca tea leaves. The orbitrap MS instrument was tuned with electrospray ionization in positive-ion mode (*m/z* 304.2). Furthermore, the optimal DART temperature setting was determined on stainless steel mesh as well as on spiked blank hair and authentic drug user hair. Figure S2 shows signal intensities of *m/z* 304.1543 for the tested settings ranging from 50 to 500 °C for analysis on mesh and from 50 to 300 °C for analysis on hair. Hair could not be analyzed at temperature settings above 300 °C, corresponding to approximately 135 °C at the sample, due to singeing of the hairs at higher temperature settings. On mesh, the temperature setting profile shows an increase in detected peak area up to 150 °C, followed by a slight decrease (Figure S2A). More detailed investigation of the temperature settings between 50 and 100 °C showed a steep increase toward the maximum detected peak area starting from 100 °C (Figure S2B). Compared to the temperature setting profile of THC,[49] ionization of cocaine occurred at a lower temperature setting. This was expected due to cocaine's higher volatility compared to THC. The temperature setting profile on spiked blank hair and authentic drug user hair is very different from that on mesh: a higher temperature setting is needed to desorb cocaine from hair resulting in an optimum temperature plateau starting from 200 and 250 °C for, respectively, spiked blank hair and drug user hair (Figure S2C and S2D). In addition to the signal intensity of cocaine, formation of thermal degradation products at the elevated temperatures in DART analysis was investigated. It was found that a minor amount of the spiked cocaine standard on mesh was converted into the main metabolite of cocaine, benzoylecgonine (BZE, *m/z* 290.1387). Since with electrospray ionization (ESI)-MS analysis no BZE was detected, it can be stated that the detected BZE was not an impurity present in the cocaine standard but was formed during DART analysis. Cocaine is known to be converted into BZE by heat,[55,56] and therefore it is most likely that the observed formation of BZE is caused by the elevated temperatures during DART analysis. The relative amount of BZE detected compared to cocaine increased with temperature, from 0.06% at 90 °C up to 0.56% at 500 °C (Figure S2A and S2B). Since at the optimal temperature setting for THC,[49] 250 °C, also an optimum cocaine signal was obtained and only 0.43% of the cocaine present was converted into BZE, this setting was chosen for all succeeding cocaine experiments.

Required mass resolution for intact hair scanning

Direct analysis of blank hair samples yields a great variety of ions. To avoid false positives, the use of HRMS was found to be crucial. Regarding cocaine, for example, many isobaric ions at *m/z* 304 were detected even in blank hair. Figure S3 shows the total ion current (TIC) of three hair scans with extracted ion chronograms (EIC) for, respectively, a 1 mass unit window and a 5 ppm mass tolerance, in correspondence with the calibration stability, around the *m/z* of cocaine: 304.1543. Two hair scans originate from blank hair samples (Figure S3A and S3B) and one from a cocaine user’s hair sample (Figure S3C). The use of a 1 mass unit window resulted in false positive results for the blank hair samples, indicating drug use with a similar temporal profile as the TIC, while a 5 ppm mass tolerance shows no cocaine signal in the blank hair. Also, the drug user hair sample showed an identical profile as the TIC in the 1 mass unit window, while the exact *m/z* of cocaine was detected only in a specific time segment of the hair sample. Upon zooming in on *m/z* 304, many other ions were detected (Figure S3D), some deviating only a few hundredths of a mass unit from the exact *m/z* of cocaine. Mass resolution of at least 60,000 FWHM would be required to be able to distinguish between a specific compound of interest and other ions formed during analysis of an intact hair sample. Consequently, instruments and imaging setups like time-of-flight (TOF)-SIMS using mass analyzers with low mass resolution[9] are not able to differentiate between a targeted compound like cocaine and other isobaric constituents resulting from blank hair analysis.

Preliminary validation of DART hair scan method for analysis of cocaine

To be able to use the DART hair scan method in forensic applications, the quantitative performance of the developed method on an orbitrap MS instrument was tested, and the method was validated against a reference LC–MS/MS analysis at a UKAS ISO 17025 accredited laboratory. Analysis of blank hair samples spiked with different secondary cocaine standard dilutions by use of a TLC sprayer revealed good correlation (R^2 = 0.979) after correction for the volume error caused by the spray technique. Similar to most other ambient ionization techniques, no internal standard can be added into the sample matrix, in this case the internal regions of the hair, and therefore no absolute quantification was possible. Instead, DART-HRMS responses of blank hair samples spiked with secondary cocaine standard were externally calibrated versus LC–MS/MS reference data. Blank hair samples as such and spiked with different diluted secondary cocaine standards through a 2 mm slit were analyzed. No cocaine was detected in the blank hair samples by either the DART hair scan method (Figure 1A) or the reference LC–MS/MS analysis, while the spiked samples yielded LC–MS/MS-detected cocaine concentrations of 1.0 ±0.4 ng/mg of hair per ppm cocaine in the spiked solution. The high standard deviation is caused by variance in the absolute amount applied onto the hair by the TLC sprayer. The lowest level cocaine solution resulting in a distinguishable peak by the DART hair scan method on an orbitrap MS instrument (Figure 1B) was 1000 times diluted secondary standard, corresponding to 0.04 ppm of cocaine. DART-HRMS analysis of hair spiked with this 1000 times diluted secondary standard yielded a signal of ~460 area counts/mm of hair scanned, and following the results of the reference analysis, this is equivalent to approximately 0.04 ng/mg of hair. This is more than a factor of 10 below the SoHT cut-off value for cocaine of 0.5 ng/mg;[20] thus the DART hair scan method on an orbitrap MS instrument is amenable for use in forensic applications. The use of a more advanced quadruple−orbitrap MS resulted in more than 10 times improved sensitivity, enabling detection of pictogram per milligram levels. Blank hair spiked with 10 times higher cocaine concentration would result in a cocaine concentration on hair around the cut-off, approximately 0.4 ng/mg, and yielded a signal on a orbitrap MS as shown in Figure 1C (~5.3 × 10^3 area counts/mm of hair). Since spiked blank hair samples are different from authentic drug user hair samples, the ability to detect cut-off concentrations in hair was confirmed by analysis of an authentic drug user hair sample containing 0.5 ng/mg cocaine following reference LC–MS/MS analysis (Figure 1D, ~4.7 × 10^3 area counts/mm of hair). Analysis of this drug user hair sample resulted in a very similar DART-HRMS response compared to blank hair spiked with 0.4 ng cocaine/mg: 4.7×10^3 and 5.3×10^3 area counts/mm of hair, respectively. Analysis of additional authentic drug user hair samples originating from a total of 10 different individuals is described below.

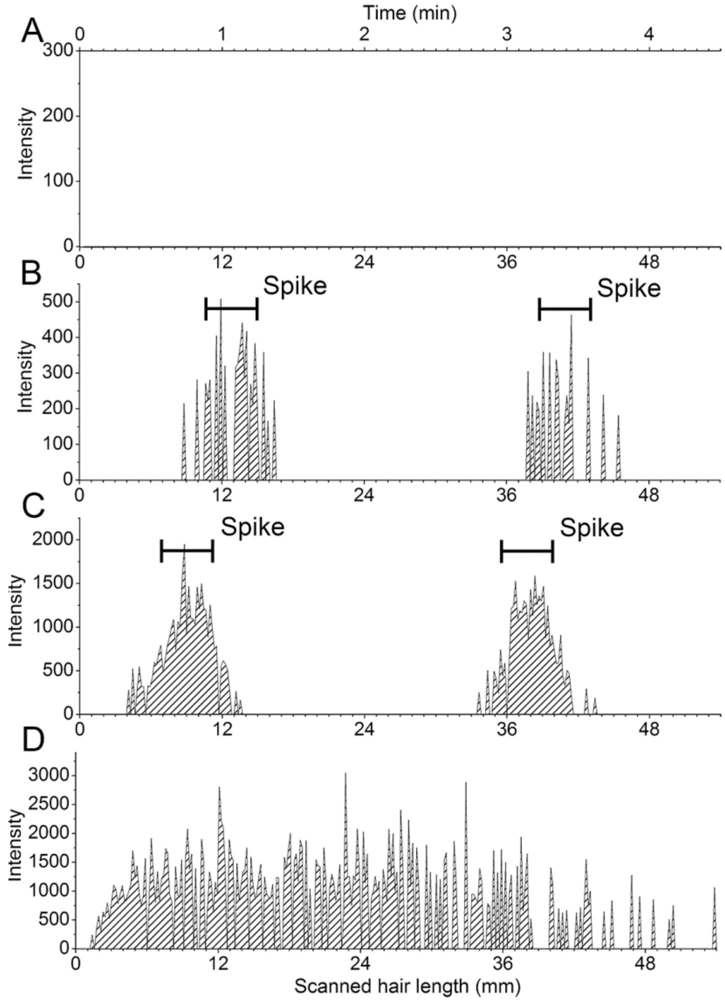

Figure 1. *Extracted ion chronograms of cocaine (m/z 304.1543) in DART-orbitrap MS longitudinal hair scans of A) blank hair, B) blank hair spiked at two zones with approximately 0.04 ng of cocaine/mg, C) blank hair spiked at two zones with approximately 0.4 ng of cocaine/mg by use of a TLC nebulizer, and D) a hair sample obtained from drug user 1 with a confirmed concentration of 0.5 ng of cocaine/mg.*

Data-dependent product ion scan events

The use of DART quadruple−orbitrap MS resulted in improved sensitivity, and additionally, it enabled sequential acquisition of high-resolution full-scan accurate mass data and data-dependent product ion scans. For this purpose, the following drugs of abuse were selected on the basis of declared usage of drug users who donated hair samples and availability of standards: cocaine, THC, amphetamine, and MDMA. First, different normalized collision energies (NCE) were tested to yield optimal product ion spectra, that is, yielding around 10% of the intact quasi-molecular ion. After recording of product ion spectra of standard solutions by direct injection ESI and use of Dip-it tips for DART ionization (Figure S4), the *m/z* values of the parent ions were used to build an inclusion list for data-dependent product ion scan acquisition. A loop count of 1 and a dynamic exclusion of 5.0 s were used to ensure a full scan every second and to maintain a sufficient number of untargeted full-scan data acquisitions along the hair and, for example, avoiding any loss of temporal drug profile resolution. The characteristic product ions of cocaine, THC, amphetamine and MDMA obtained by DART-MS/HRMS are listed in Table 1 and are in good agreement with ESI product ions found in literature.[57-59] Furthermore, product ion ratios were calculated on the basis of intensities of ions after DART-MS/HRMS analysis of blank hair spiked with standards, as listed in Table 1. It has to be noted that no distinction could be made between THC and cannabidiol (CBD): both compounds have the same elemental composition and yield the same product ions. CBD, however, is present in cannabis at very low concentrations, and therefore the majority of the detected ions will originate from THC. A DART hair scan of a blank hair sample spiked on two 2 mm zones with methanolic solutions containing 5 ppm of cocaine and amphetamine and of THC and MDMA, respectively, mimicking two short periods of different heavy drug use, is shown in Figure 2: the top panel shows the full HRMS scans, while the panels below show the presence of characteristic product ions in the data-dependent product ion scans at the respectively spiked zones only. The use of data-dependent product ion scans had no significant influence on the sensitivity of the full-scan measurements ($P > 0.05$).

Table 1. *Drugs of abuse and related ions,[a] theoretical and experimental exact masses, and product ion intensity ratios[b]*

Ion	Theor *m/z*	Exptl *m/z*	Δ (mDa)	Ratio spiked on blank hair	Ratio drug user samples
			Cocaine		
$[M+H]^+$	304.1543	304.1553	+1.0		
$[M+H–C_6H_5COOH]^+$	182.1176	182.1180	+0.4	23	31
$[M+H–C_6H_5COOH–CH_4O]^+$	150.0913	150.0918	+0.5		
			THC		
$[M+H]^+$	315.2319	315.2328	+0.9		
$[M+H–C_4H_8]^+$	259.1693	259.1700	+0.7	0.42	0.26
$[M+H–C_9H_{14}]^+$	193.1223	193.1229	+0.6		
			Amphetamine		
$[M+H]^+$	136.1121	136.1123	+0.2		
$[M+H–NH_3]^+$	119.0855	119.0860	+0.5	0.27	0.32
$[C_7H_7]^+$	91.0542	91.0543	+0.1		
			MDMA		
$[M+H]^+$	194.1176	194.1181	+0.5		
$[M+H–CH_5N]^+$	163.0754	163.0756	+0.2	14	12
$[M+H–C_3H_9N]^+$	135.0441	135.0444	+0.3		

[a]*Monitored by data-dependent product ion scans via DART-quadrupole orbitrap MS/HRMS.* [b]*Detected for spiked blank hair and authentic drug user samples.*

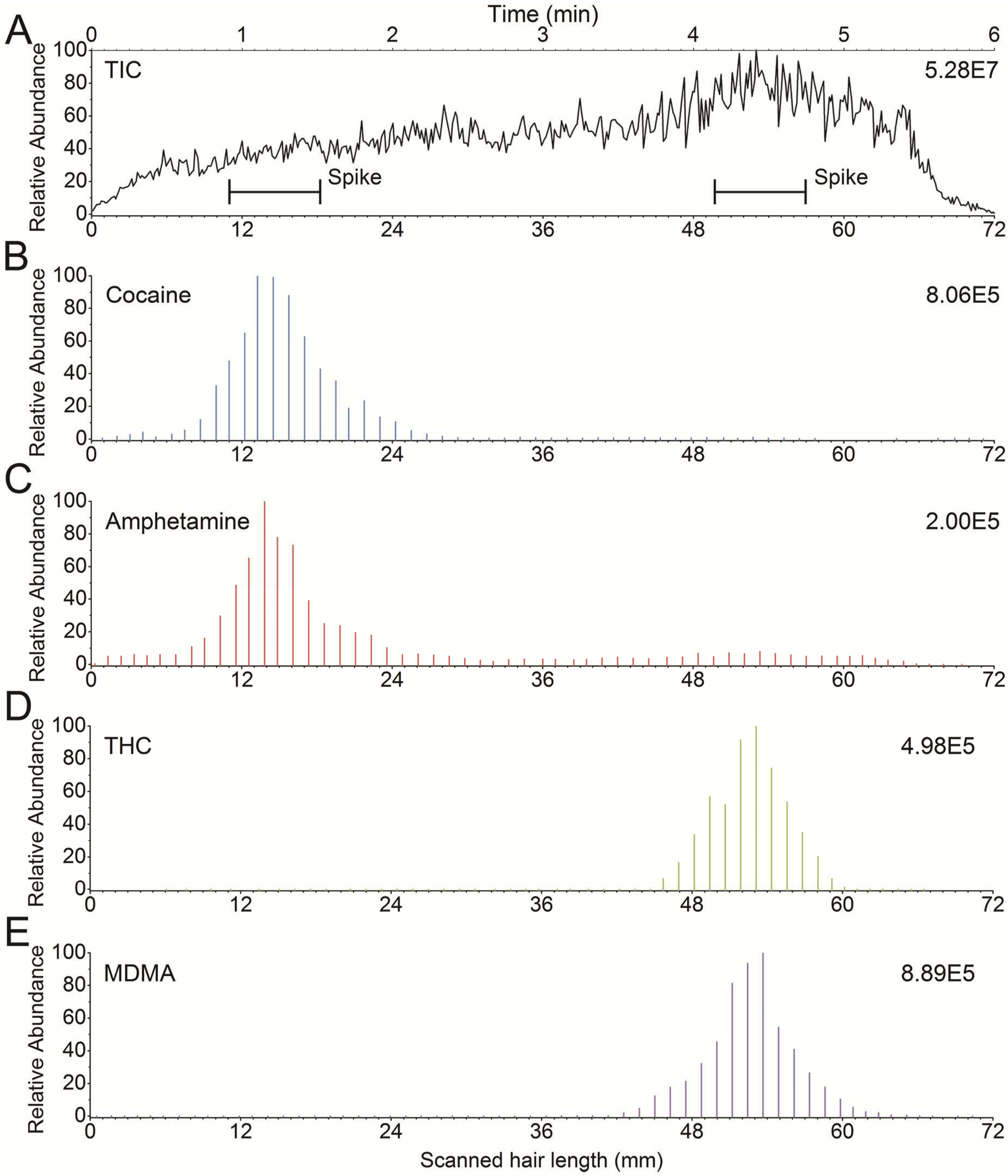

Figure 2. *DART-quadrupole orbitrap MS/HRMS hair scan of blank hair spiked at two zones with a solution of 5 ppm of cocaine and amphetamine on the first zone and 5 ppm of THC and MDMA on the second zone, showing A) total ion current (TIC) of full-scan data acquisition events, B) extracted ion chronograms (EIC) of m/z 105.0335 + 150.0913 + 182.1177 in cocaine data-dependent product ion scans, C) EIC of m/z 91.0542 + 119.0855 in amphetamine data-dependent product ion scans, D) EIC of m/z 193.1223 + 259.1693 in THC data-dependent product ion scans, and E) EIC of m/z 135.0441 + 163.0754 in MDMA data-dependent product ion scans.*

Drug user hair analyses

Authentic drug user hair samples from 10 different drug users have been analyzed by the developed hair scan method with data-dependent product ion acquisition for cocaine, THC, amphetamine, and MDMA. The presence of these compounds in drug user hair samples was confirmed by comparing the ratio of their characteristic product ions in the triggered data-dependent product ion scans with the ratios calculated from standards spiked on blank hair (Table 1). For this purpose, an ion ratio tolerance of 50% was used as recommended in recent literature following the analysis of 120 pesticides and comparison of the ion ratio variation against different legal and guidance documents.[60] By use of these criteria, six of the authentic drug user hair samples tested positive for cocaine (seven declared), three for THC (seven declared), two for amphetamine (four declared), and two for MDMA (three declared) (Table 2). Not all results were in agreement with the declared drug use (Table 2). Self-declared drug usage, however, is known to be unreliable due to underreporting or overreporting by users.[61,62] Different temporal drug concentration profiles could be obtained for individual compounds by a simple DART hair scan analysis, as shown for example in Figure 3 for drug user 7. Cocaine was detected all along the hair sample in high concentrations, which analytically suggests continuous and stable cocaine use, while amphetamine and MDMA were detected in variable concentrations, suggesting an increased use of amphetamine during the last month and of MDMA between 0.5 and 1.5 month before sampling. However, please note that the translation of analytical profiles to actual retrospective drug use may not be straightforward due to secondary mechanisms like incorporation into the hair matrix from sebum or sweat[2,15] and delayed release from tissue.[2,24] Besides DART analysis, subsamples of the drug user hair sample shown in Figure 3 were used for reference LC–MS/MS analysis, both before and after decontamination. The detected cocaine concentrations were 74.8 and 62.1 ng/mg, respectively, before and after dichloromethane decontamination. The two analyzed wash solvents contained 13.7 and 1.2 ng of cocaine/mg as expressed in hair levels. In addition to cocaine, amphetamine, and MDMA were detected by LC–MS/MS in the drug user hair sample (48.5 and 3.8 ng/mg), which was in perfect qualitative agreement with the DART hair scan results (Table 2 and Figure 3).

Table 2. *Drugs of abuse detected in drug user hair samples[a] and self-declared drug use of individual drug users.*

Drug user	Detected drugs of abuse by data-dependent product ion scans	Additional compounds detected by retrospective data analysis	Self-reported drug use[b]
1	Cocaine	Nicotine	Cocaine, MDMA
2	Cocaine	Zolpidem, nicotine, PMMA	Cocaine, cannabis, MDMA, amphetamine, GHB, ketamine
3	-	Nicotine	Cocaine
4	-	Nicotine	Cocaine, cannabis
5	Cocaine, MDMA	Methoxetamine, nicotine	Cocaine, cannabis, MDMA, methoxetamine
6	Cocaine, THC	Hydromorphone, methadone, methoxetamine, nicotine, cotinine	Cocaine, cannabis, amphetamine, GHB, methoxetamine, 2C-B, 4-FMP, benzodiazepine
7	Cocaine, amphetamine, MDMA	Oxycodone, BZE, hydroxycocaine, norbuprenorphine, nicotine, cotinine	Cocaine
8	THC	Nicotine	Cannabis
9	Amphetamine	Norbuprenorphine, caffeine, nicotine	Cannabis, amphetamine
10	Cocaine, THC	Hydroxy-THC, ketamine, caffeine, nicotine, cotinine	Cannabis, amphetamine, LSD, ketamine

[a]By data-dependent product ion scans via DART-quadrupole orbitrap MS/HRMS. [b]GHB, γ-hydroxybutyrate; 2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 4-FMP, 4-fluoroamphetamine; LSD, lysergic acid diethylamide.

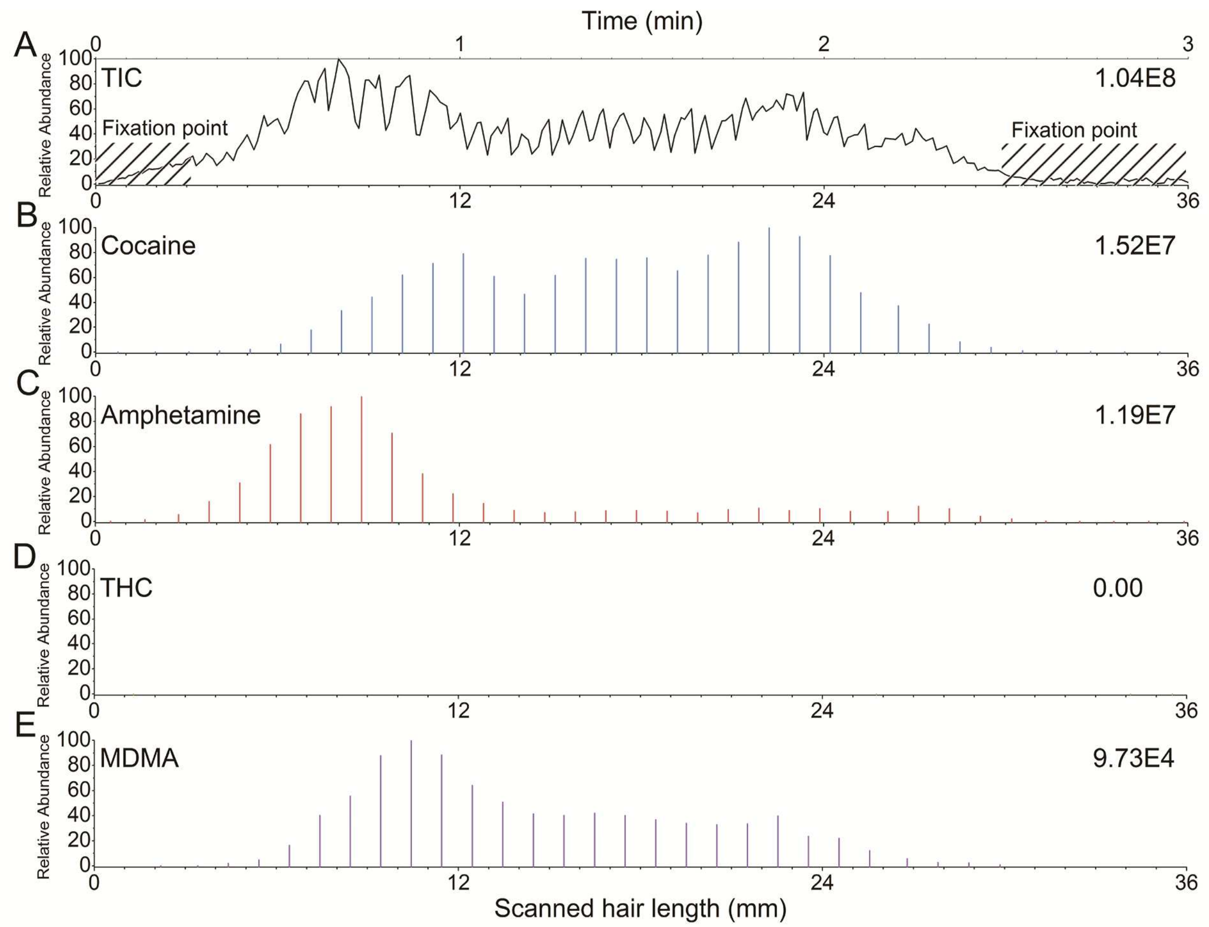

Figure 3. *DART-quadrupole orbitrap MS hair scan of a hair sample obtained from drug user 7 showing A) TIC of full-scan data acquisition events, B) EIC of m/z 105.0335 + 150.0913 + 182.1177 in cocaine data-dependent product ion scans, C) EIC of m/z 91.0542 + 119.0855 in amphetamine data-dependent product ion scans, D) EIC of m/z 193.1223 + 259.1693 in THC data-dependent product ion scans, and E) EIC of m/z 135.0441 + 163.0754 in MDMA data-dependent product ion scans.*

Cocaine metabolite detection

Metabolite detection is an important tool to confirm drug use and avoid false positives caused by external contamination. The main metabolite of cocaine is BZE, which is formed by hydrolytic ester cleavage resulting in a carboxylic acid. Where the major metabolite of THC, 11-nor-9-carboxy-THC (THC-COOH),[63] is incorporated in very low concentrations (cut-off value = 0.0002 ng/mg),[20] BZE is incorporated in higher amounts with a SoHT recommended cut-off value of 0.05 ng/mg, compared to 0.5 ng/mg for cocaine.[20] Since cocaine could be detected with sufficient sensitivity, it was anticipated that BZE could be detected as well in drug user hair samples with high cocaine content. As discussed in the section describing DART-HRMS optimization, only a very small part of the cocaine, less than 0.5%, was found to be converted into BZE at the elevated temperature used during DART analysis. BZE was detected in drug user hair samples of a daily cocaine user (user 7) with a BZE to cocaine signal ratio above 10% (Figure S5). Retrospective data analysis, as discussed in more detail below, was used to screen for several other cocaine metabolites (Table S1) and yielded one additional hit: hydroxycocaine. Detection of BZE and hydroxycocaine shows that incorporated metabolites of cocaine could be detected by DART in authentic hair samples, but careful evaluation of the BZE to cocaine ratio is needed to avoid false positive results due to the minimal conversion of cocaine into BZE during DART analysis.

Retrospective interrogation of full scan accurate mass data

While the data-dependent analysis described above focuses on four specific drugs of abuse, the full-scan data acquired in the same longitudinal hair scan can be used for retrospective data analysis for other psychoactive substances and occasionally abused pharmaceutical drugs present in the hair. An extensive list of more than 100 drugs of abuse and related compounds was compiled from literature: Table S1 shows the full list of investigated compounds with their chemical formulas and expected *m/z* values. The list was used for retrospective analysis of the full-scan data obtained from DART-HRMS scanning of drug user hair samples with a mass tolerance of 5.0 ppm, resulting in several positive hits. Next to commonly used compounds like nicotine and caffeine, also additional psychoactive substances and occasionally abused pharmaceutical drugs were found: hydromorphone, ketamine, methadone, methoxetamine, norbuprenorphine, oxycodone, *p*-methoxymethylamphetamine (PMMA), and zolpidem. Detection of ketamine and methoxetamine was in good correlation with drug use as declared by the drug users (Table 2). Different temporal drug profiles within hair samples from drug users were observed, indicating multiple drug use at different time points during several months (Figure S6). Most prominent is the increased cocaine signal in the hair sample between 11 and 20 mm, indicating higher cocaine use approximately between 1 and 2 months prior to sampling. In addition, three metabolites were detected: BZE and hydroxycocaine, as discussed before, and the nicotine metabolite cotinine. Even though cotinine is forensically not interesting, the detection thereof is another confirmation that analysis of endogenous incorporated metabolites is possible via the DART hair scan method. Hydroxy-THC was detected in the hair of one drug user stating regular cannabis use; unfortunately, it was not possible to distinguish whether this hydroxy-THC was 11-OH-THC (a known metabolite of THC)[64] or a different hydroxy-THC.

Ultrafast untargeted DART screening

In addition to obtaining DART hair scans of drug user hair samples, DART-HRMS could also be used for ultrafast untargeted screening of hair samples without longitudinal scanning, thereby sacrificing the retrospective timeline assessment possibility. For this purpose, hair was introduced directly into the ionization region with tweezers (Figure S7A). The resulting procedure is somewhat comparable to the use of Dip-it tips for solutions: within a minute, a hair sample could be screened for multiple drugs of abuse by obtaining full-scan high-resolution data. Figure S7B shows an example of ultrafast untargeted screening of a cocaine user hair sample (drug user 7) introduced twice into the DART ionization region in just over half a minute, resulting in two clearly distinguishable rises of the TIC. The obtained full-scan HRMS data were retrospectively interrogated as described above, by use of the list of compounds in Table S1. Several compounds, in very good correspondence with the DART hair scan and reference LC–MS/MS analysis, were detected exclusively at data points corresponding to measurement of the hair, including cocaine, BZE, amphetamine, and nicotine, showing the preliminary potential of DART-HRMS as a within-minute prescreening method for hair samples.

Higher spatial resolution DART analysis

The DART spot size with the standard exit cone is approximately 5 mm in diameter, resulting in a retrospective timeline accuracy of ±2 weeks.[49,65] This time resolution is already a significant improvement over the 1−3 cm segments used in conventional hair analysis, which correspond to approximately 1−3 months of hair growth. Despite the fact that the distribution of a drug along the hair length following administration is highly compound-dependent,[2,10,15,66] further improvement of the DART spot size to a shorter time frame could still be relevant to obtain more accurate timelines of drug administration in specific forensic cases. To achieve a lower spot size, an improved high spatial resolution DART exit cone with an internal diameter of 0.5 mm, compared to the 2.5 mm internal diameter standard exit cone, was tested. Multiple locks of blank hair were spiked through 2 and 1 mm slits with 25 times diluted secondary cocaine standard and analyzed by use of either the standard or the high-resolution exit cone (Figure S8). The DART spot size decreased from 5.1 ±1.1 mm with the standard exit cone to 3.3 ±1.0 mm with the high-resolution exit cone. Use of the high spatial resolution exit cone would therefore result in a retrospective timeline accuracy of about 10 days, if a hair growth rate of 1.0 cm/month is assumed.[10,22,25,26] Using the high spatial resolution exit cone did negatively influence the hair scan sensitivity by a factor 2−5, as could be expected from decreasing the spot size by a factor of 2.5 and the reduced gas flow when the high spatial resolution exit cone was used. It is therefore recommended to use the high spatial resolution exit cone only with an MS instrument that provides high sensitivity like, e.g., a quadruple−orbitrap MS instrument, to benefit from the improved timeline accuracy without losing too much sensitivity.

Conclusions

A fast and simple DART-(MS/)HRMS ambient hair scanning approach was successfully developed. By use of data-dependent product ion scan events together with full-scan high-resolution data acquisition, confirmation of identity of commonly used drugs of abuse in hair samples could be obtained without compromising the sensitivity of the method. Cocaine was detected at levels below the SoHT recommended cut-off value. After further validation, the method may be applied in forensic investigations. Individual temporal profiles of both targeted and untargeted drugs of abuse can be obtained within one run and without the need for time-consuming and laborious analysis of hair segments. Since the achieved retrospective timeline accuracy is around 10 days with a high spatial resolution exit cone, the DART hair scan method may become an effective alternative for traditional segmented hair analysis methods with much lower analysis times. Detection of several metabolites was achieved and could exclude external contamination. The acquired full-scan high-resolution data could be retrospectively interrogated by use of a list of more than 100 compounds and yielded multiple hits in good agreement with declared drug use, including ketamine and methoxetamine, demonstrating the value of longitudinal hair scanning in combination with full-scan data.

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Supporting information Chapter 4

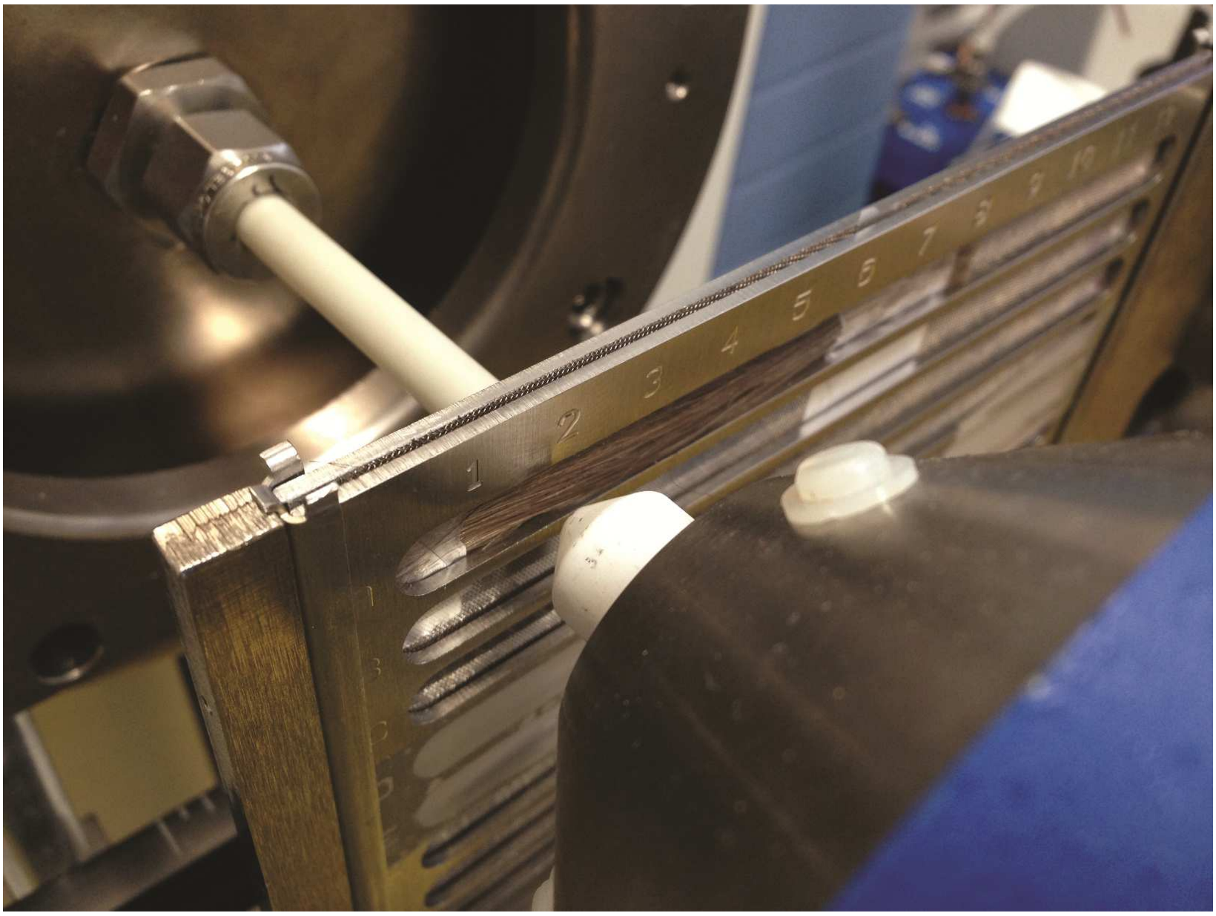

Figure S1. *Experimental set-up for the analysis of intact locks of hair using DART-orbitrap MS scanning.*

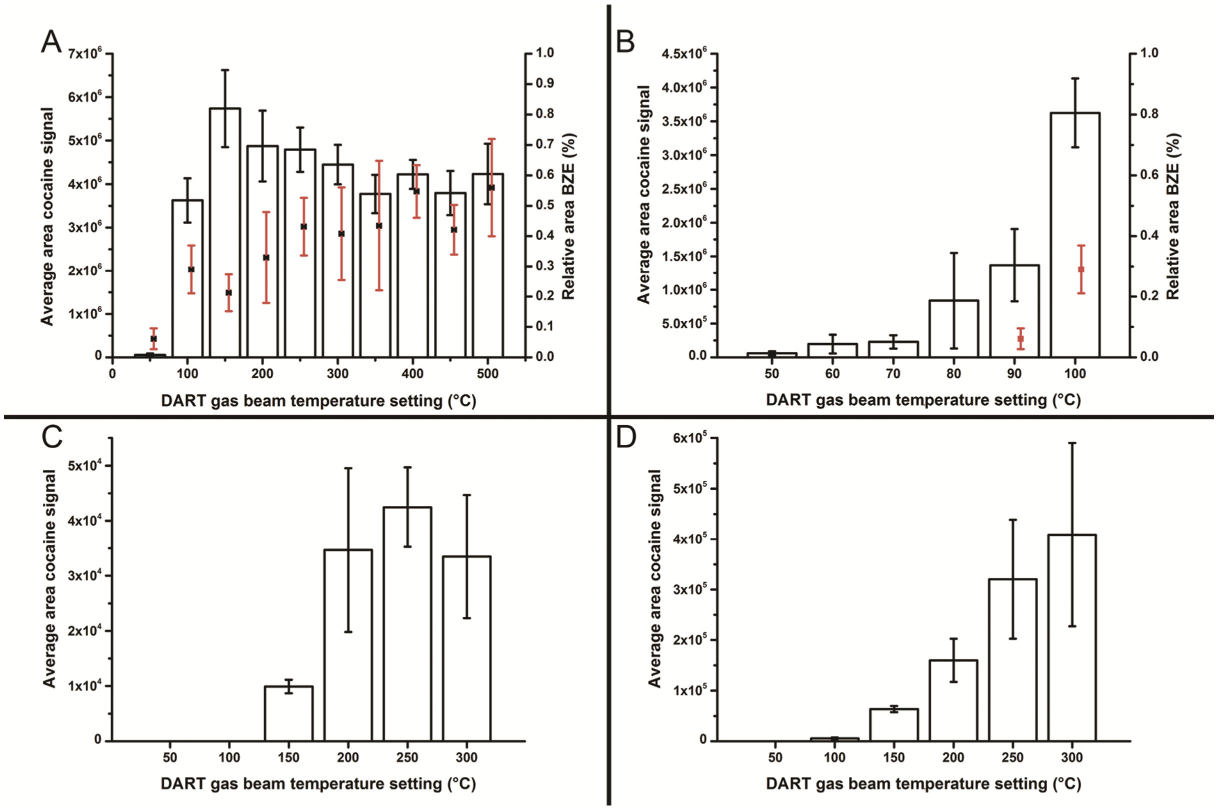

Figure S2. *Average DART-orbitrap MS peak area for cocaine (m/z 304.1543, represented by bars) and BZE peak area relative to cocaine (m/z 290.1392, represented by squares) at different DART helium temperature settings for A) and B) cocaine standard spiked on stainless steel mesh sheet, C) cocaine standard spiked on blank hair, and D) drug user hair.*

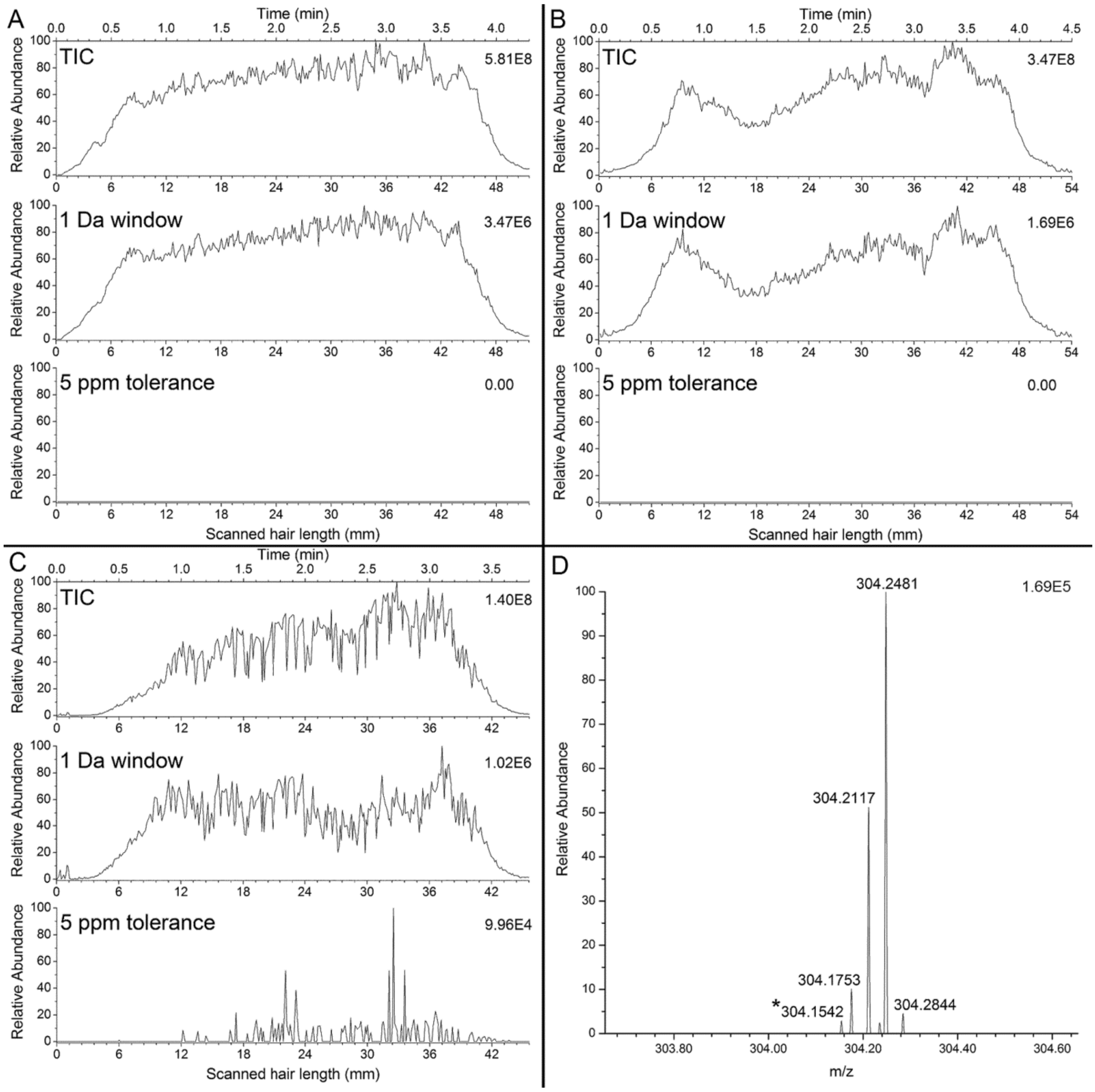

Figure S3. *DART orbitrap MS total ion current (TIC) and extracted ion chronograms (EIC) of m/z 303.6543 - 304.6543 (1 Da window) and m/z 304.1528 - 304.1558 from longitudinal hair scans of A) and B) blank hair samples and C) a hair sample obtained from a drug user, and D) mass spectrum averaged from 6 to 42 mm of the hair scan shown in C, representing a 1 Da mass window. The intact quasi-molecular ion of cocaine is indicated by an asterisk.*

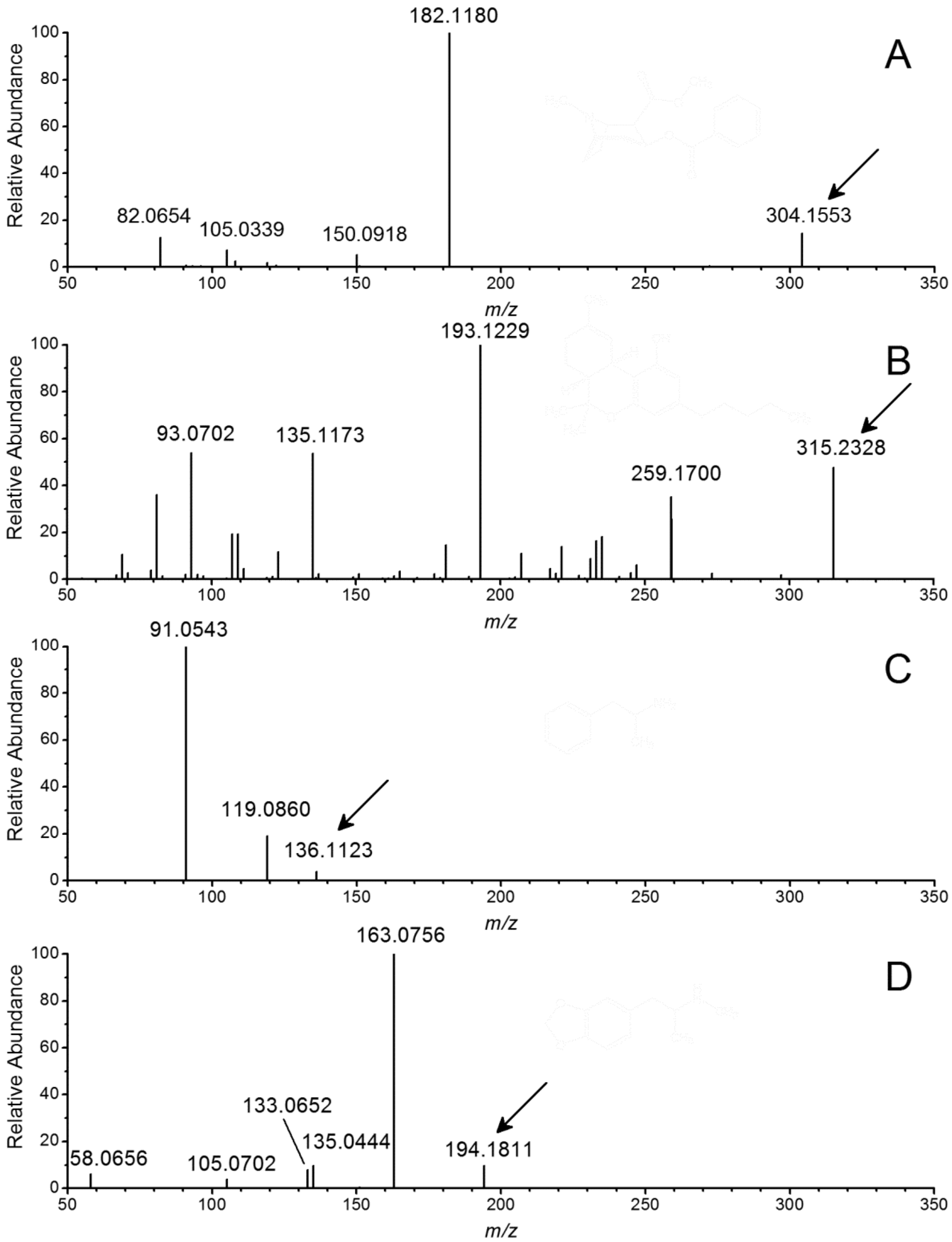

Figure S4. *DART quadrupole orbitrap MS/HRMS product ion spectra obtained by Dip-it analysis of the following solutions: A) 10 ppm cocaine (normalized collision energy (NCE)=35), B) 10 ppm THC (NCE=35), C) 10 ppm amphetamine (NCE=30), and D) 10 ppm MDMA (NCE=30). The intact quasi-molecular ions are indicated by an arrow.*

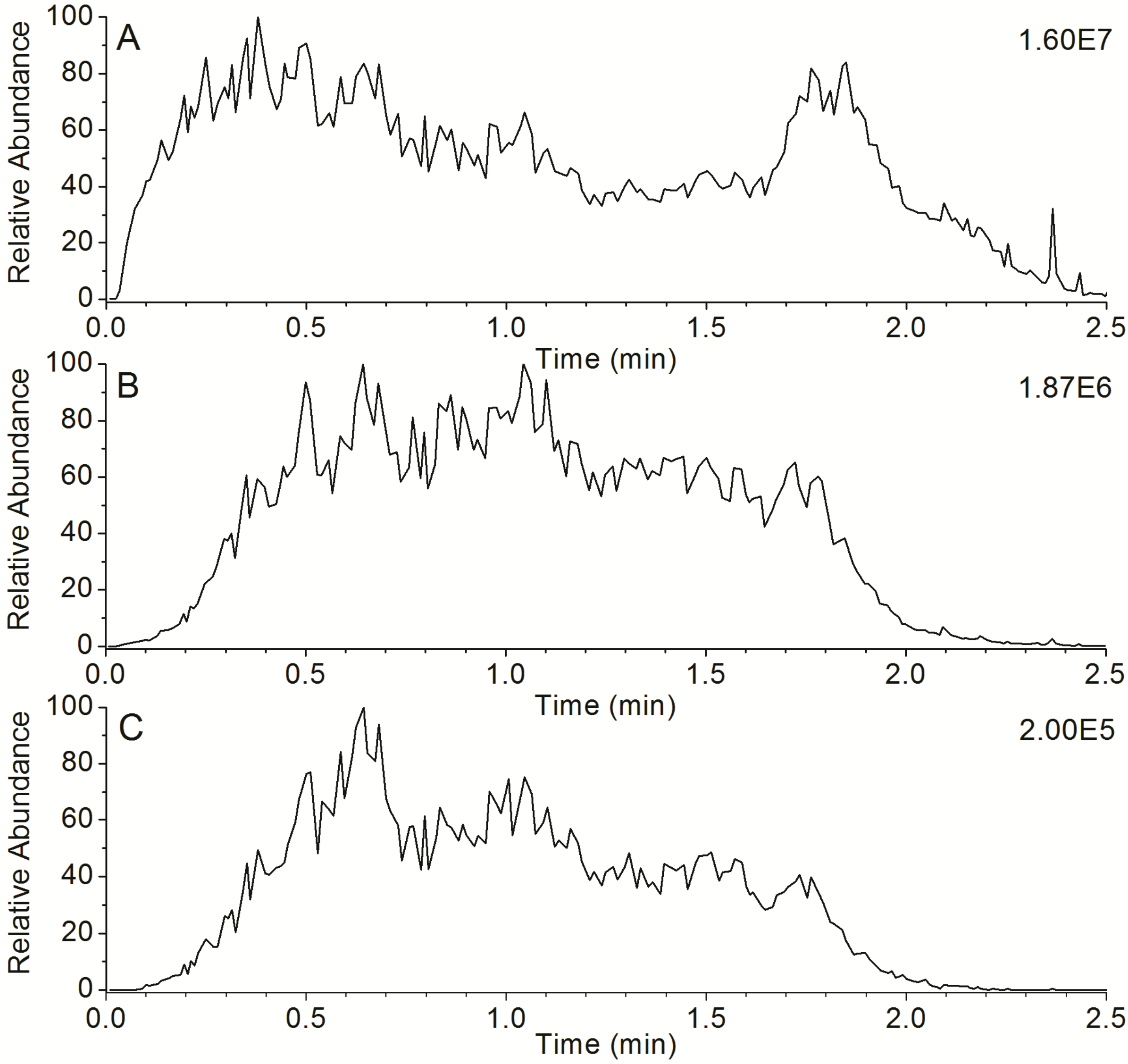

Figure S5. *A) Total ion current and extracted ion chronograms of B) cocaine, m/z 304.1543, and C) BZE, m/z 290.1387 in DART orbitrap MS longitudinal hair scan of a hair sample obtained from drug user 7.*

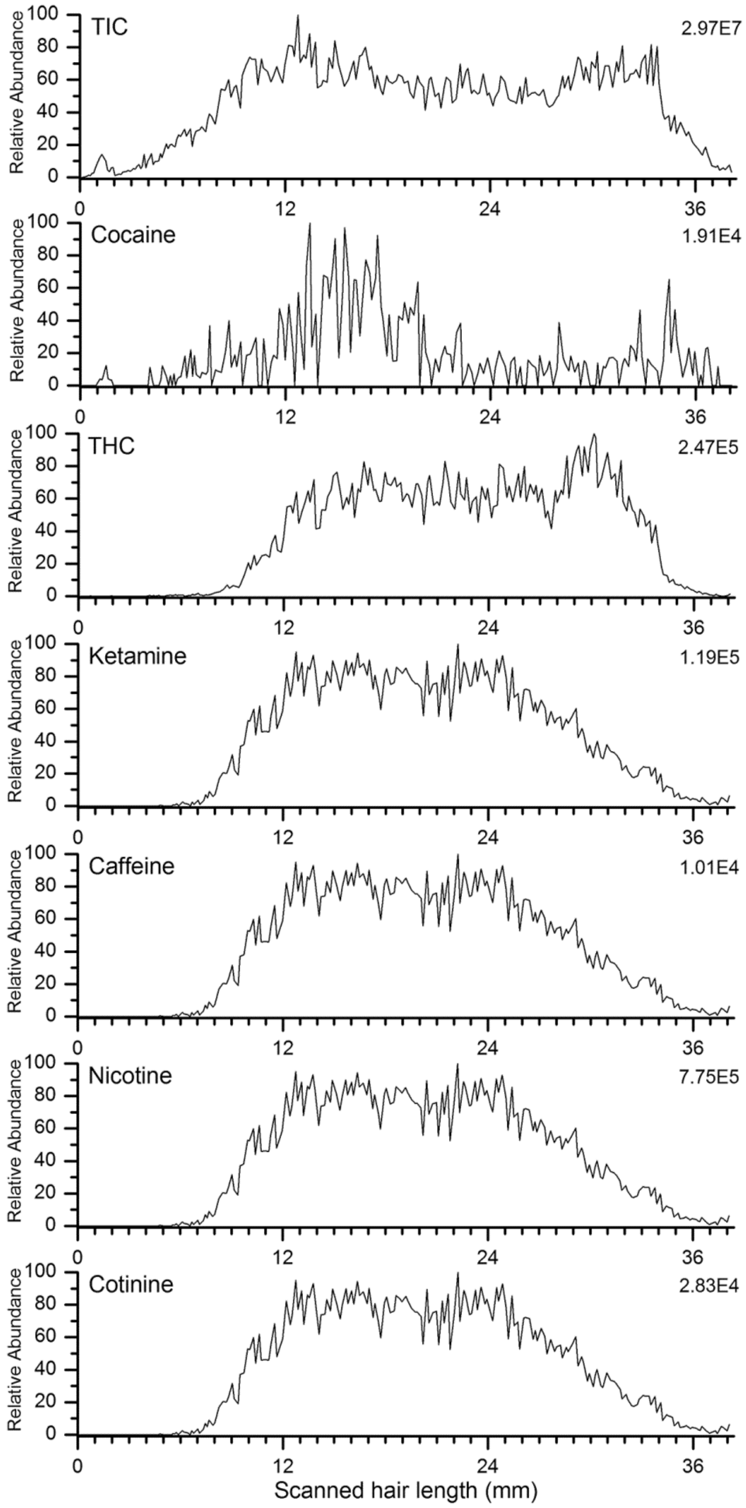

Figure S6. *Total ion current and extracted ion chronograms of cocaine (m/z 304.1543), THC (m/z 315.2324), ketamine (m/z 238.0994), caffeine (m/z 195.0877), nicotine (m/z 163.1230) and cotinine (m/z 177.1022) in DART orbitrap MS longitudinal hair scan of a hair sample obtained from drug user 10.*

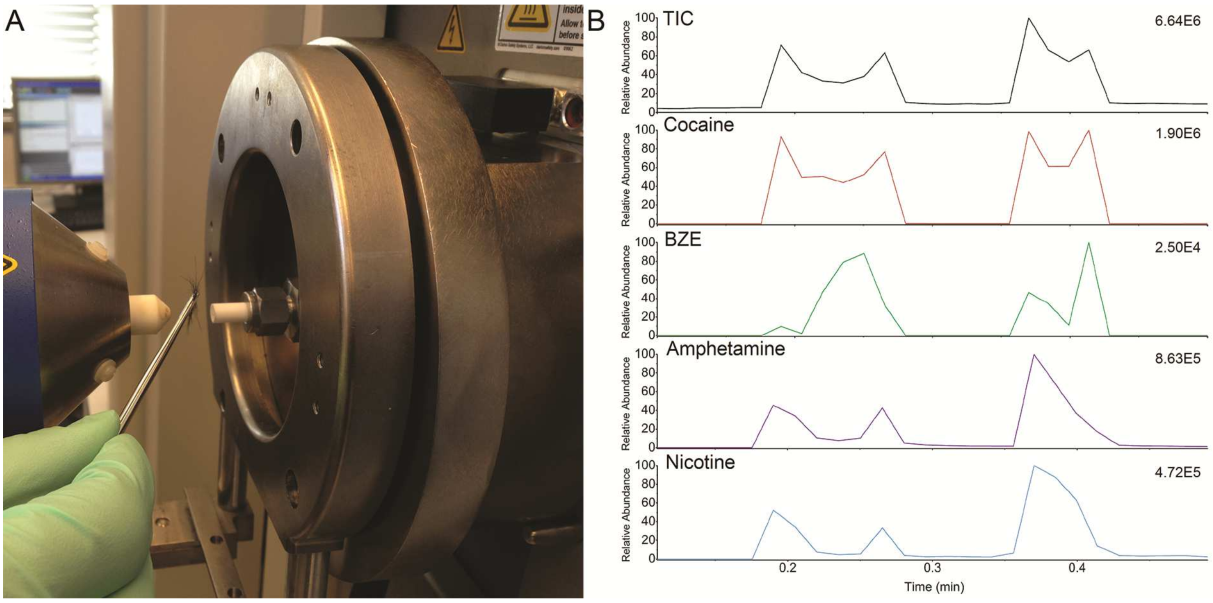

Figure S7. *Ultrafast untargeted DART screening of hair: A) experimental set-up and B) total ion current and extracted ion chronograms of cocaine (m/z 304.1543), BZE (m/z 290.1387), amphetamine (m/z 136.1121) and nicotine (m/z 163.1230) in DART orbitrap MS longitudinal hair scan of a hair sample obtained from drug user 7.*

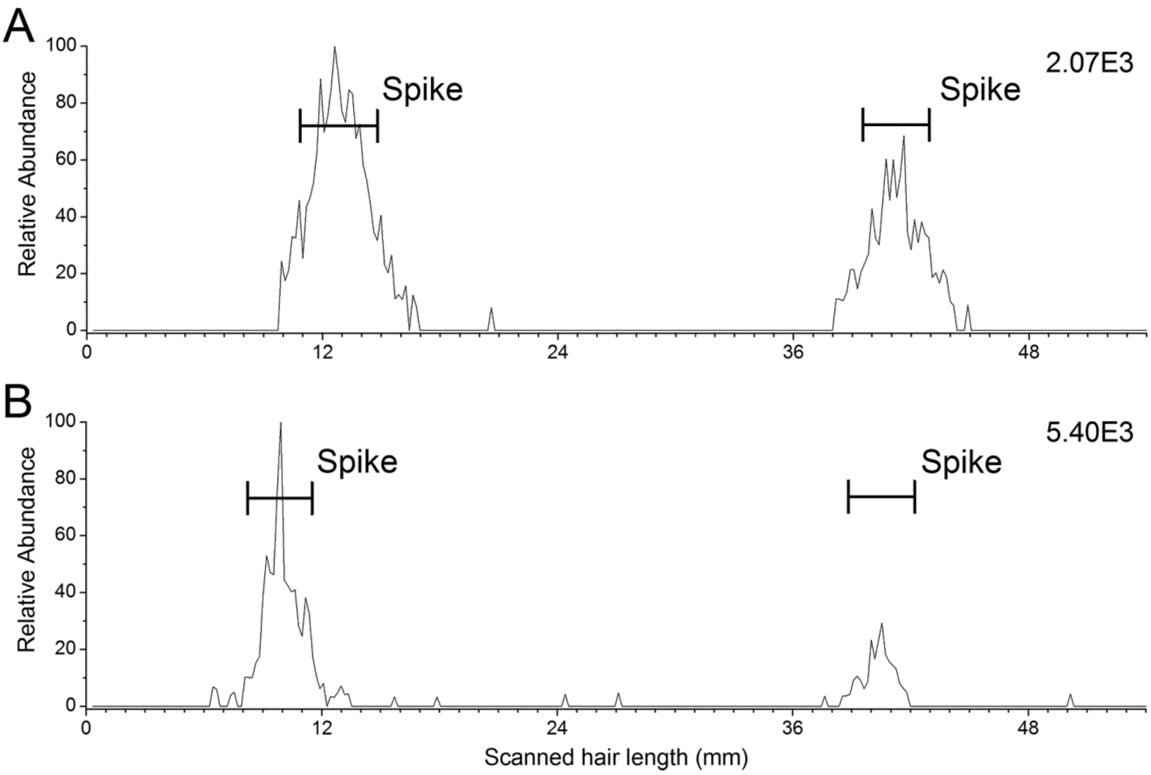

Figure S8. *Extracted ion chronograms of cocaine (m/z 304.1543) in DART orbitrap MS longitudinal hair scans of blank hair spiked through 2 and 1 mm slits using A) standard DART exit cone and B) high resolution DART exit cone.*

Table S1. *Drugs of abuse and related compounds with their exact mass used for retrospective interrogation of full scan high resolution mass data obtained by DART-HRMS hair scanning.*

Compound	Chemical formula	Exact mass $[M+H]^+$	Compound	Chemical formula	Exact mass $[M+H]^+$
THC	$C_{21}H_{30}O_2$	315.2324	Phencyclidine	$C_{17}H_{25}N$	244.2065
CBD	$C_{21}H_{30}O_2$	315.2324	Codeine	$C_{18}H_{21}NO_3$	300.1600
CBN	$C_{21}H_{26}O_2$	311.2011	Methadone	$C_{21}H_{27}NO$	310.2171
THCA-A	$C_{22}H_{30}O_4$	359.2222	EDDP	$C_{20}H_{23}N$	278.1909
THC-COOH	$C_{21}H_{28}O_4$	345.2066	Buprenorphine	$C_{29}H_{41}NO_4$	468.3114
THC-COOH-glucuronide	$C_{27}H_{36}O_{10}$	521.2387	Norbuprenorphine	$C_{25}H_{35}NO_4$	414.2644
Hydroxy-THC	$C_{21}H_{30}O_3$	331.2273	GHB	$C_4H_8O_3$	105.0552
JWH-007	$C_{25}H_{25}NO$	356.2014	GHB-glucuronide	$C_{10}H_{16}O_9$	281.0873
JWH-015	$C_{23}H_{21}NO$	328.1701	Ketamine	$C_{13}H_{16}ClNO$	238.0999
JWH-018	$C_{24}H_{23}NO$	342.1858	Norketamine	$C_{12}H_{14}ClNO$	224.0842
JWH-019	$C_{25}H_{25}NO$	356.2014	Methoxphenidine	$C_{20}H_{25}NO$	296.2014
JWH-020	$C_{26}H_{27}NO$	370.2171	Methoxetamine	$C_{15}H_{21}NO_2$	248.1651
JWH-073	$C_{23}H_{21}NO$	328.1701	Zolazepam	$C_{15}H_{15}FN_4O$	287.1308
JWH-081	$C_{25}H_{25}NO_2$	372.1964	Tiletamine	$C_{12}H_{17}NOS$	224.1109
JWH-122	$C_{25}H_{25}NO$	356.2014	Butalbital	$C_{11}H_{16}N_2O_3$	225.1239
JWH-200	$C_{25}H_{24}N_2O2$	385.1916	Propyphenazone	$C_{14}H_{18}N_2O$	231.1497
JWH-203	$C_{21}H_{22}ClNO$	340.1468	Alprazolam	$C_{17}H_{13}ClN_4$	309.0907
JWH-210	$C_{26}H_{27}NO$	370.2171	Clonazepam	$C_{15}H_{10}ClN_3O_3$	316.0489
JWH-250	$C_{22}H_{25}NO_2$	336.1964	Chlordiazepoxide	$C_{16}H_{14}ClN_3O$	300.0904
JWH-251	$C_{22}H_{25}NO_2$	336.1964	Diazepam	$C_{16}H_{13}ClN_2O$	285.0795
JWH-307	$C_{26}H_{24}FNO$	386.1920	Flunitrazepam	$C_{16}H_{12}FN_3O_3$	314.0941
JWH-398	$C_{24}H_{22}ClNO$	376.1468	Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	321.0198
HU-210	$C_{25}H_{38}O_3$	387.2899	Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	335.0354
AM-694	$C_{20}H_{19}FINO$	436.0574	Oxazepam	$C_{15}H_{11}ClN_2O_2$	287.0587
AM-1220	$C_{26}H_{26}N_2O$	383.2123	Nitrazepam	$C_{15}H_{11}N_3O_3$	282.0879
AM-2201	$C_{24}H_{22}FNO$	360.1764	Nordazepam	$C_{15}H_{11}ClN_2O$	271.0638
AM-2232	$C_{24}H_{20}N_2O$	353.1654	Temazepam	$C_{16}H_{13}ClN_2O_2$	301.0744
MAM-2201	$C_{25}H_{24}FNO$	374.1920	Triazolam	$C_{17}H_{12}Cl_2N_4$	343.0517
RCS-4	$C_{21}H_{23}NO_2$	322.1807	Zolpidem	$C_{19}H_{21}N_3O$	308.1763

RCS-8	$C_{25}H_{29}NO_2$	376.2277	Zopiclone	$C_{17}H_{17}ClN_6O_3$	389.1129
UR-144	$C_{21}H_{29}NO$	312.2327	Caffeine	$C_8H_{10}N_4O_2$	195.0882
XLR-11	$C_{21}H_{28}FNO$	330.2233	Nicotine	$C_{10}H_{14}N_2$	163.1235
PB-22	$C_{23}H_{22}N_2O_2$	359.1760	Cotinine	$C_{10}H_{12}N_2O$	177.1028
5-F-PB-22	$C_{23}H_{21}FN_2O_2$	377.1665	PMMA	$C_{11}H_{17}NO$	180.1388
WIN 48,098	$C_{23}H_{26}N_2O_3$	379.2022	Atropine	$C_{17}H_{23}NO_3$	290.1756
WIN 55,212-2	$C_{27}H_{26}N_2O_3$	427.2022	Scopolamine	$C_{17}H_{21}NO_4$	304.1549
Cocaine	$C_{17}H_{21}NO_4$	304.1549	Mephedrone	$C_{11}H_{15}NO$	178.1232
Benzoylecgonine	$C_{16}H_{20}NO_4$	290.1392	4-MEC	$C_{12}H_{17}NO$	192.1388
Ecgonine methyl ester	$C_{10}H_{17}NO_3$	200.1287	3-MMC	$C_{11}H_{15}NO$	178.1232
Cocaethylene	$C_{18}H_{23}NO_4$	318.1705	Pentedrone	$C_{12}H_{17}NO$	192.1388
Ecgonine	$C_9H_{15}NO_3$	186.1130	6-APB	$C_{11}H_{13}NO$	176.1075
Norcocaine	$C_{16}H_{19}NO_4$	290.1392	Paroxetine	$C_{19}H_{20}FNO_3$	330.1505
Anhydroecgonine methyl ester	$C_{10}H_{15}NO_2$	182.1181	3,4-DMMC	$C_{12}H_{17}NO$	192.1388
Hydroxycocaine	$C_{17}H_{21}NO_5$	320.1492	4-FA	$C_9H_{12}FN$	154.1032
Amphetamine	$C_9H_{13}N$	136.1126	4-MA	$C_{10}H_{15}N$	150.1283
Methamphetamine	$C_{10}H_{15}N$	150.1283	Benzylpiperazine	$C_{11}H_{16}N_2$	177.1392
MDA	$C_{10}H_{13}NO_2$	180.1025	Buphedrone	$C_{11}H_{15}NO$	178.1232
MDMA	$C_{11}H_{15}NO_2$	194.1181	Butylone	$C_{12}H_{15}NO_3$	222.1130
MDEA	$C_{12}H_{17}NO_2$	208.1338	Cathine	$C_9H_{13}NO$	152.1075
Morphine	$C_{17}H_{19}NO_3$	286.1443	MBDB	$C_{12}H_{17}NO_2$	208.1338
Ethylmorphine	$C_{19}H_{23}NO_3$	314.1756	4-MTA	$C_{10}H_{15}NS$	182.1003
Heroin	$C_{21}H_{23}NO_5$	370.1654	Naphydrone	$C_{19}H_{23}NO$	282.1858
6-(M)AM	$C_{19}H_{21}NO_4$	328.1549	Pentylone	$C_{13}H_{17}NO_3$	236.1287
3-MAM	$C_{19}H_{21}NO_4$	328.1549	2C-B	$C_{10}H_{14}BrNO_2$	260.0286
Hydrocodone	$C_{18}H_{21}NO_3$	300.1600	4-FMP	$C_9H_{12}FN$	154.1032
Hydromorphone	$C_{17}H_{19}NO3$	286.1443	LSD	$C_{20}H_{25}N_3O$	324.2076
Oxycodone	$C_{18}H_{21}NO_4$	316.1549	N-demethyl LSD	$C_{19}H_{23}N_3O$	310.1919
Oxymorphone	$C_{17}H_{19}NO_4$	302.1392			

Chapter 5

Critical Comparison of Mass Analyzers for Forensic Hair Analysis by Ambient Ionization Mass Spectrometry

This chapter is submitted as:

W.F. Duvivier, T.A. van Beek, M.W.F. Nielen. Critical Comparison of Mass Analyzers for Forensic Hair Analysis by Ambient Ionization Mass Spectrometry. *Rapid Communications in Mass Spectrometry*

Abstract

RATIONALE: Recently, several direct and/or ambient mass spectrometry (MS) approaches have been suggested for drugs of abuse imaging in hair. The use of mass spectrometers with insufficient selectivity could result in false-positive measurements due to isobaric interferences. Different mass analyzers have been evaluated regarding their selectivity and sensitivity for the detection of Δ^9-tetrahydrocannabinol (THC) from intact hair samples using direct analysis in real time (DART) ionization.

METHODS: Four different mass analyzers namely 1) an orbitrap, 2) a quadrupole orbitrap, 3) a triple quadrupole, and 4) a quadrupole time-of-flight (QTOF) were evaluated. Selectivity and sensitivity were assessed by analyzing secondary THC standard dilutions on stainless steel mesh screens and blank hair samples, and by the analysis of authentic cannabis user hair samples. Additionally, separation of isobaric ions by use of travelling wave ion mobility (TWIM) was investigated.

RESULTS: The use of a triple quadrupole instrument resulted in the highest sensitivity, however, transitions used for multiple reaction monitoring were only found to be specific when using high mass resolution product ion measurements. A mass resolution of at least 30,000 FWHM was necessary to avoid overlap of THC with isobaric ions originating from the hair matrix. Even though selectivity was enhanced by use of TWIM, the QTOF instrument in resolution mode could not indisputably differentiate THC from endogenous isobaric ions in drug user hair samples.

CONCLUSIONS: Only the high resolution of the (quadrupole) orbitrap instruments and the QTOF instrument in high resolution mode distinguished THC in hair samples from endogenous isobaric interferences. As expected, enhanced selectivity compromises sensitivity and THC was only detectable in hair from heavy users.

Introduction

In forensic toxicology, hair can be used as an alternative matrix to body fluids for the detection of drugs of abuse, pharmaceuticals, and other compounds of interest. Analysis of hair samples can reveal the exposure of an individual to drugs over a longer period of time.[1] Routine hair analysis methods involve extensive sample preparation followed by gas chromatography or liquid chromatography (tandem) mass spectrometry (GC− or LC−(MS)/MS).[2] Over the last decade, MS imaging methods using matrix-assisted laser desorption/ionization (MALDI),[3-14] laser ablation electrospray ionization (LAESI),[15] or metal assisted-secondary ion mass spectrometry (MetA-SIMS)[14,16] have been proposed for the analysis of single hairs utilizing the excellent spatial resolutions of these ionization techniques. The analysis of single hairs, however, can yield inconsistent results caused by asynchronous hair growth cycles between individual hairs.[6,10,17-19] Recently, a new hair analysis method using direct analysis in real time (DART) ionization to desorb and ionize analytes from intact locks of hair was developed.[20,21] Total analysis time was reduced tremendously compared to the extensive sample preparation used in conventional hair analysis methods and analysis of a complete lock of hair, consisting of 150-200 hairs, eliminates the difficulties associated with asynchronous hair growth.[20,21] By the use of simultaneous full-scan high-resolution data acquisition and data-dependent product ion scan events, untargeted detection of multiple psychoactive substances and occasionally abused pharmaceutical drugs was demonstrated and structural confirmation of four commonly used drugs of abuse was achieved.[21]

On the one hand, direct and/or ambient MS for analysis of hair samples eliminates the need of extensive sample preparation and allows acquisition of continuous timelines of drug exposure. On the other hand, a drawback of direct ionization techniques is the relatively high standard deviation of the measurements causing difficulties for quantification.[22-24] In addition, direct analysis of a sample can cause ionization of unwanted compounds from the hair sample matrix. The background signal originating from unwanted ionization can overlap with the compounds of interest, as previously described for the analysis of cocaine in hair samples. Blank hair samples yielded six different endogenous isobaric ions at *m/z* 304, which could only be differentiated by use of a mass analyzer with mass resolution of at least 60,000 full width at half maximum (FWHM).[21] Furthermore, it was previously envisioned that use of a triple quadrupole mass spectrometer could increase the sensitivity of the DART hair scan method,[20] however, this has not been demonstrated yet. With DART as ionization technique, cleaner spectra are generally observed in comparison with other direct or ambient MS techniques. For example, desorption electrospray ionization (DESI) and MALDI yield ionization of, respectively, many small and larger polar compounds and MALDI matrix, which could interfere with compounds of interest.

In this work, the selectivity and sensitivity of ambient MS is critically evaluated for the analysis of Δ^9-tetrahydrocannabinol (THC), the psychoactive compound in cannabis, by a comparison of four mass spectrometers with different mass analyzers. Next to the previously reported (quadrupole) orbitrap instruments, yielding full-scan high-resolution data,[20,21] triple quadrupole and quadrupole time-of-flight (QTOF) instruments are investigated with respect to their selectivity and sensitivity. Full-scan data is obtained using the orbitrap and QTOF instruments, while the triple quadrupole instrument is used in multiple reaction monitoring (MRM) mode. In addition, travelling wave ion mobility (TWIM) for the separation of interfering isobaric ions is explored. TWIM enables millisecond separation of gas phase ions based on their molecular size and shape.[25,26] The selectivity and sensitivity of each mass analyzer is determined by the analysis of a secondary THC standard on mesh and blank hair samples. Finally, the instrument selectivity is critically compared for the analysis of authentic cannabis user hair samples.

Experimental

Chemicals and materials. Methanol (HPLC gradient grade) was purchased from J.T. Baker Chemicals (Deventer, The Netherlands). Stainless steel mesh screens (opening size 0.24 × 0.24 mm) were purchased from IonSense (Saugus, MA, USA).

Secondary THC standard and dilutions. A secondary THC standard was used for the spiking experiments due to legal restrictions. For this purpose, 2.4 g crushed cannabis plant material (Cannabis sativa) was extracted for 30 min using 100 mL of methanol. After filtration over filter paper (Schleicher & Schuell, Dassel, Germany: No. 595; diameter 70 mm), the resulting secondary THC standard was stored in a glass bottle at 7 °C. The THC concentration was determined using a previously described LC–MS/MS method[20] and found to be 235 parts per million (ppm). The secondary THC standard was diluted using methanol to yield concentrations ranging from 0.008 to 2.4 ppm.

Hair samples. Blank hair samples were obtained from non-drug users. Hair of two chronic cannabis users was sampled anonymously in the city of Wageningen (The Netherlands) to obtain authentic cannabis user hair samples. The volunteers used cannabis at least three times a week in the six months prior to sampling, and informed consent was obtained.

Sensitivity experiments on mesh. The sensitivity of DART ionization coupled to the different mass spectrometers was investigated using secondary THC standard dilutions. For this purpose, 2.0 µL of the dilutions was pipetted onto stainless steel mesh screens. This resulted in a square spiked zone of approximately 30 mm2 on mesh. Twelve zones of different dilutions (4 concentration levels in triplicate) were pipetted next to each other with 0.5 cm distance in between the zones.

Spiking of blank hair samples. Blank hair samples were spiked by use of a thin-layer chromatography (TLC) sprayer as described before.[20] In short, the blank hair samples were covered by a 1 mm thick stainless steel mask with different slits (1 × 10 or 2 × 10 mm). A dilution of the secondary THC standard was then briefly (<1 s) sprayed using the TLC sprayer onto blank hair samples from a distance of approximately 10 cm. Exposure of only a small longitudinal section of the hair enabled accurate spiking of small zones of the hair with secondary THC standard.

DART hair scan sample preparation and analysis. Prior to analysis, drug user hair samples were decontaminated using a single methanol wash followed by a single aqueous SDS solution wash. After a Milli-Q water rinsing step, the hair samples were left to dry for 2 h.[27] The analysis of spiked blank and authentic cannabis user hair samples was as described before.[20,21] In short, an intact lock of hair (approximately 150-200 hairs and 10 mg/cm) was attached to a stainless steel mesh screen using adhesive tape. The mesh screen was then placed inside a DART X-Z module holder (IonSense), which had slits of 113 mm width × 7 mm height. Spiked mesh and hair samples were longitudinally scanned at a scan speed of 0.2 mm/s using a DART-SVP ion source (IonSense). The DART-SVP source was positioned 5 mm from the sample, which was 3 mm from the ceramic inlet tube of the mass spectrometer and was operated using helium as ionization gas (flow rate of ~3.7 L/min), with a temperature setting of 250 °C in positive-ion mode.

Orbitrap MS settings. An Exactive orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used to obtain full-scan measurements with a scan range of *m/z* 100.0−1000.0 in positive mode, a mass resolution of 100,000 FWHM, and a maximum injection time of 100 ms. The capillary temperature was 275 °C and the capillary voltage was 25 V.

Quadrupole orbitrap (tandem) MS settings. A Q-Exactive quadrupole orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific) was used to obtain full-scan measurements with a scan range of *m/z* 100.0-1000.0 in positive mode, a mass resolution of 140,000 FWHM, and a maximum injection time of 100 ms. The capillary temperature was 275 °C and the capillary voltage was 25 V. Product ion scans of THC were obtained with an isolation width of 1.0 Da and a normalized collision energy (NCE) of 25.

Triple quadrupole MS settings. A Xevo TQ-S MS system (Waters, Milford, MA, USA) was used in MRM mode using a collision energy of 30 eV to monitor the following ion transitions: *m/z* 315.3 to *m/z* 259.2 and 193.2. The source temperature was 120 °C and the cone voltage was 35 V.

TOF (tandem) MS settings. A Synapt G2-S MS system (Waters) was used to obtain full-scan measurements in positive mode with a scan range of *m/z* 50-1200 in both resolution ('V' optics, approximately 20,000 FWHM) and high resolution mode ('W' optics, approximately 40,000 FWHM). The source temperature was 150 °C and the cone voltage was 40 V. TWIM experiments were performed using an ion mobility cell nitrogen gas flow of 90 mL/min, a wave velocity of 650 m/s and a wave height of 40 V. QTOF MS data were post-acquisition corrected on the $[M+H]^+$ ion of THC at *m/z* 315.2319. Product ion scans of THC were obtained with a precursor-ion resolution of 32,000 and a trap collision energy of 25 eV.

Results and Discussion

Comparison of sensitivity of the detection of THC on mesh

The sensitivity of DART coupled to the different mass spectrometers was first evaluated by pipetting dilutions of the secondary THC standard onto mesh to avoid bias originating from the hair matrix. The triple quadrupole instrument was operated in MRM mode, monitoring the transition of THC to the two most abundant fragments (*m/z* 259.3 and 193.2, representing the loss of, respectively, C4H8 and C9H14). For the QTOF instrument, two modes were tested: resolution mode (20,000 FWHM) and high resolution mode (40,000 FWHM). The effect of the QTOF resolution mode on the sensitivity was investigated. The lowest detectable concentration was determined via different protocols due to differences in the obtained raw data from the mass spectrometers. For the orbitrap instruments, the noise when using a 5 ppm mass tolerance, in correspondence with the calibration stability, is zero and thus no signal-to-noise ratios could be determined. Instead, the lowest concentration at which a continuous peak could be obtained (Figure S1A and S1B) was designated as the lowest detectable concentration. For the triple quadrupole and QTOF instruments, the lowest detectable concentration was defined as a peak having a signal-to-noise ratio of ten, which enables clear distinction of the peak from the noise (Figure S1C and S1D). Since the triple quadrupole instrument was operated in MRM mode, the total of both transitions was used for the determination of the lowest detectable concentration. The settings and results for each instrument are displayed in Table 1. As expected, the triple quadrupole instrument has the best sensitivity for the detection of THC spiked on mesh, with an almost three times higher sensitivity than the quadrupole orbitrap instrument and approximately eight times higher sensitivity than the QTOF instrument in resolution mode. When using the high resolution mode of the TOF mass analyzer, i.e., ‘W’ optics instead of ‘V’ optics, an approximately threefold signal loss was observed compared with the resolution mode. As previously reported,[20] the older orbitrap instrument was less sensitive than the quadrupole orbitrap instrument. In addition to the lowest detectable concentration, a calibration curve could be obtained by analysis of different dilutions of the secondary THC standard (Figure S2). For all instruments, good linearity was achieved ($R^2 \geq 0.990$, Table 1). The relative standard deviation of the measurements (n=6 for each concentration level) was found to be between 25 and 36%, except for the triple quadrupole instrument yielding an average RSD of 56%. The relatively high standard deviations observed in this study are in good agreement with values reported in literature for direct DART ionization without internal standard.[22-24]

Table 1. *Performance of different mass spectrometers used in this research.*

MS	Mass analyzer	Mass resolution (FWHM)	Lowest detectable absolute THC amount on mesh (ng)*	R^2 (0 – 4.7 ng)	Average RSD
Thermo Exactive	Orbitrap	100,000	0.47	0.990	25%
Thermo Q-Exactive	Quadrupole orbitrap	140,000	0.052	0.997	35%
Waters TQS	Triple quadrupole	Nominal	0.019	0.996	56%
Waters Synapt G2-S	QTOF - Resolution mode	20,000	0.16	0.996	36%
	- High resolution mode	40,000	0.47	0.995	32%

* All instruments operated in full-scan mode, except for the triple quadrupole instrument

Selectivity comparison for the detection of THC in hair samples

Following up on the detection of THC spiked on mesh, secondary THC standard was also spiked onto blank hair samples using the TLC spray technique described before, resulting in small longitudinal zones of THC on the hair samples.[20] DART ionizes not only the compounds of interest, but many more compounds present in the sample matrix or the surrounding environment. When zooming in at the exact mass of THC in an orbitrap DART hair scan at *m/z* 314.7-315.7, several isobaric ions were observed (Figure 1B) that could cause false-positive hair analysis results when using mass spectrometers with insufficient resolution. These ions were not detected when analyzing secondary THC standard pipetted onto mesh (Figure 1A), indicating that the isobaric ions originate from the hair matrix and not from the lab environment nor the instrument set-up. The same phenomenon was previously reported for the analysis of cocaine in hair, where a mass resolution of at least 60,000 FWHM was required to differentiate between cocaine and isobaric ions from the blank hair matrix.[21] The minimum resolution for analysis of THC from hair samples using DART-MS was calculated from the isobaric ions shown in Figure 1B. To be able to separate THC from the other ions, a resolution of at least 30,000 FWHM is required. The needed resolution was confirmed with the use of the QTOF instrument in resolution and high resolution mode (Figures 1C and 1D). The resolution mode (20,000 FWHM) is not sufficient to baseline separate THC from the isobaric ions, while the resolution in high resolution mode (40,000 FWHM) is sufficient.

Since a triple quadrupole instrument has nominal mass resolution, the use of full-scan measurements was not considered for DART hair scanning using this instrument. Instead, MRM mode to monitor collision induced ion transitions was investigated regarding its selectivity. As described in the Experimental section, two transitions were initially used: *m/z* 315.3 to *m/z* 259.2 and 193.2. Figure 2A shows a spiked blank hair sample analyzed using the triple quadrupole instrument. It can be noted that, for both transitions, the noise originating from blank hair, analyzed between approximately 0.5 and 3.9 min, is much higher than the baseline signal, from 0.0 to 0.5 min and from 3.9 to 4.2 min. The high background signal from the hair matrix makes a triple quadrupole instrument using these transitions useless for DART hair scanning, unless the signal obtained from drug user hair sample is always significantly above the background signal. For this purpose, many different blank and drug user hair samples should be analyzed to establish a threshold to differentiate THC originating from drug use from the background signal.

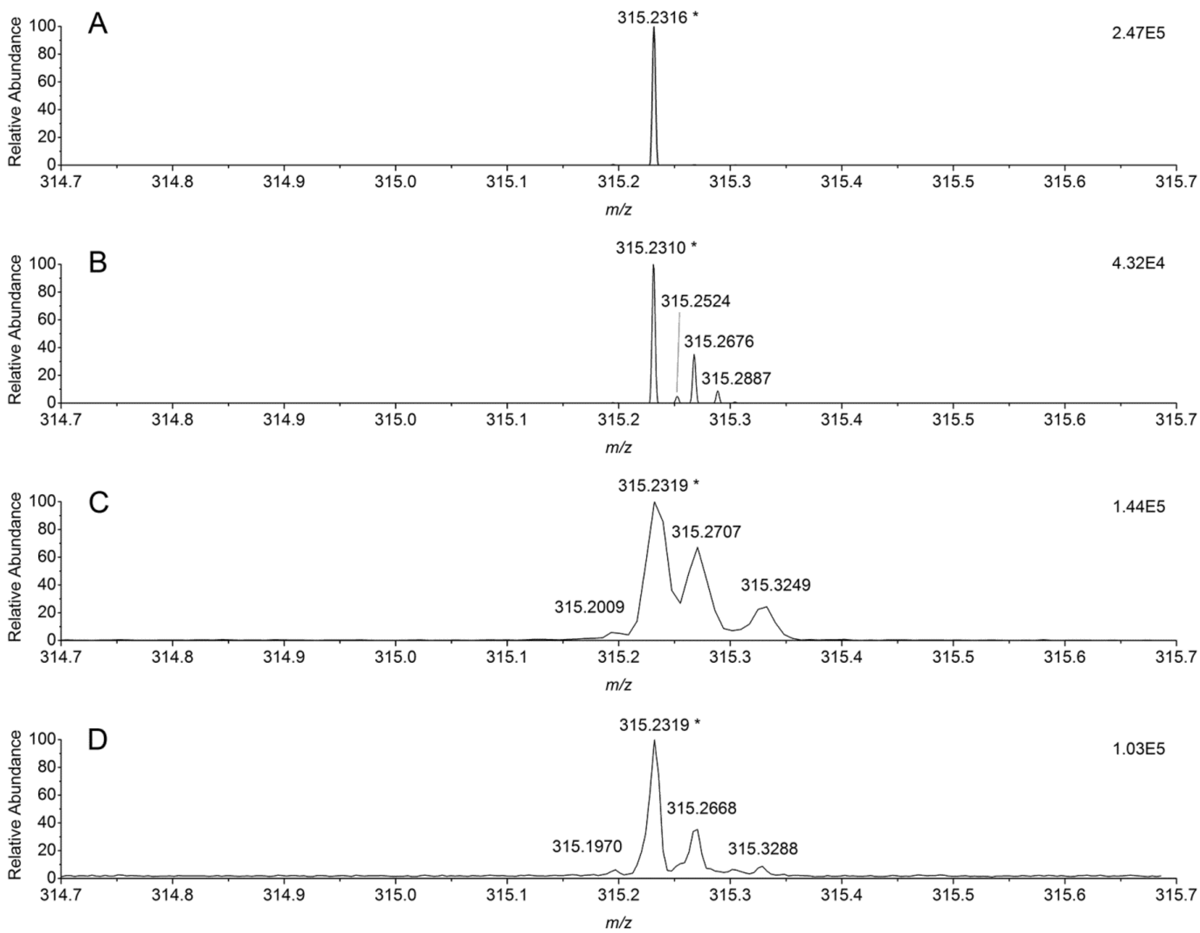

Figure 1. *A) DART quadrupole orbitrap MS spectrum of secondary THC standard pipetted on mesh, B) DART quadrupole orbitrap MS spectrum of secondary THC standard spiked on blank hair, C) DART-QTOF MS spectrum in resolution mode of secondary THC standard spiked on blank hair, and D) DART-QTOF MS spectrum in high resolution mode of secondary THC standard spiked on blank hair. The $[M+H]^+$ ion of THC is indicated by an asterisk.*

The specificity of the used transitions was further investigated using quadrupole orbitrap and QTOF instruments. The *m/z* of the quasi-molecular ion of THC with a mass window of 1 Da was selected for fragmentation, and the resulting fragment ions were detected with high resolution using the orbitrap and TOF mass analyzers. The product ion spectra of blank hair spiked with secondary THC standard and blank hair as such are shown in Figure S3. When blank hair was analyzed using the described quadrupole orbitrap settings, no ions with the exact mass of THC nor of the two fragments used during triple quadrupole analysis were detected (Figure 2B), indicating that these transitions are specific for THC provided the product ions are detected at a tolerance window of 5 ppm. When a 1 Da window was applied (Figure 2C), only some low intensity spikes of the *m/z* 259 and 193 fragment were observed during the complete measurement. These low intensity spikes do not explain the background signal observed in the triple quadrupole measurements. However, differences in fragmentation spectra can occur since the higher-energy collisional dissociation (HCD) cell fragmentation of the quadrupole orbitrap instrument is energetically different from the collision-induced dissociation (CID) fragmentation of the triple quadrupole instrument.[28] MS/MS on the QTOF instrument (Figure 2D and 2E) indeed showed higher intensities for the *m/z* 259 and 193 fragments when applying a 1 Da window, while only spikes of these fragments were observed when a 50 ppm tolerance window, in correspondence with the calibration stability without the use of an internal lock mass, was used. The results shown in Figure 2D and 2E indicate that the transitions used on the triple quadrupole instrument are more specific when the mass resolution is sufficient. As an alternative to the selected MS/MS ion transitions towards *m/z* 259.2 and 193.2, the use of two other THC product ions, *m/z* 135.1168 and 93.0699 was considered, but these were found to be even less specific since the analysis of blank hair yielded ions with the same exact *m/z* along the entire hair (Figure 2B and 2D, and Figure S3B). Selectivity of the used ion transitions was also theoretically assessed. Theoretical calculation of the probability that any other (endogenous) compound would yield the same precursor and product ions as selected for THC showed that the used transitions are not selective enough according to the threshold criteria described by Berendsen et al.[29], in accordance with the experimental results discussed in this section. Even though the triple quadrupole showed the highest sensitivity, from the above it was concluded that the use of a triple quadrupole instrument for ambient MS hair scanning is not feasible for THC due to the insufficient selectivity of product ions.

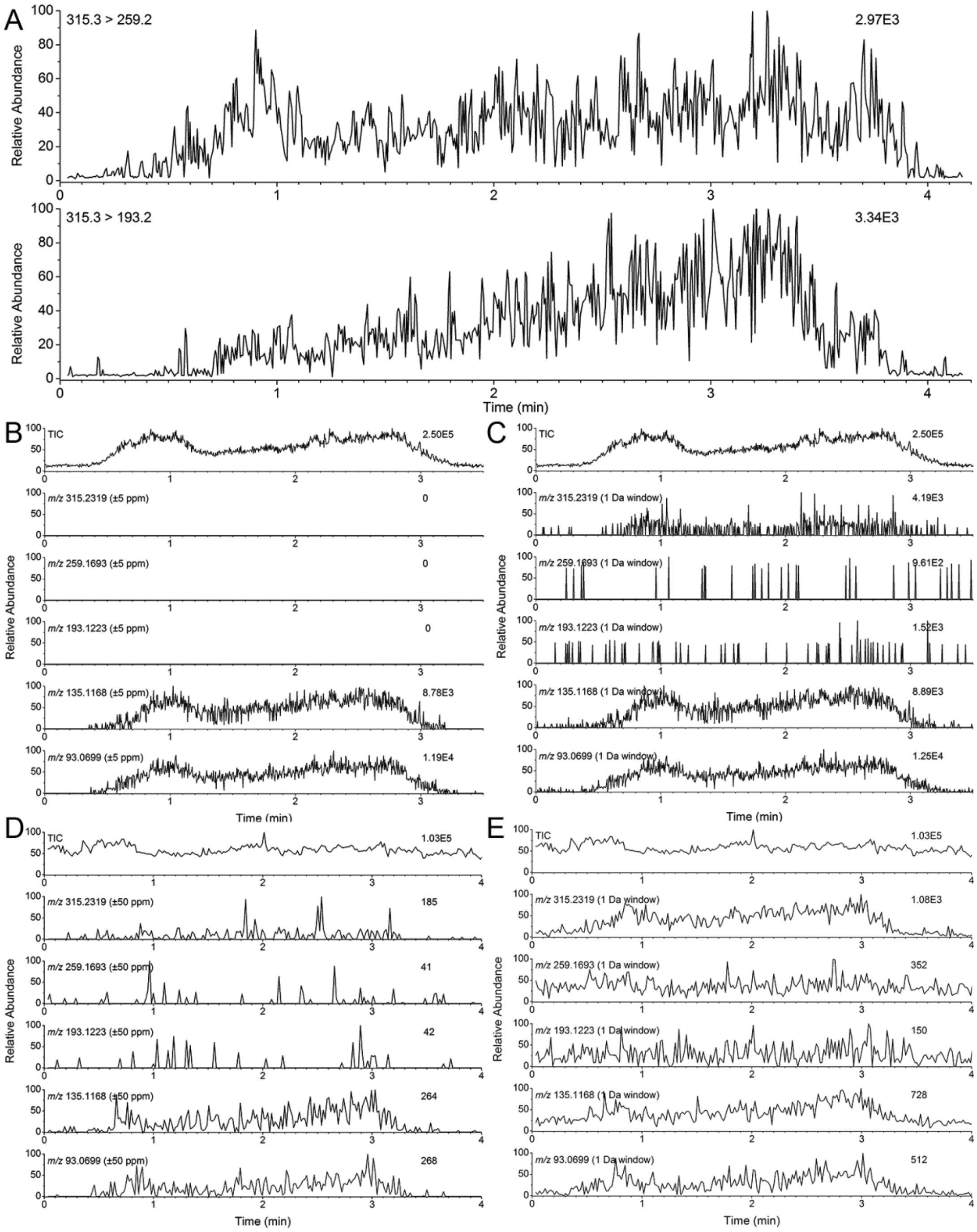

Figure 2. *A) DART-triple quadrupole MRM measurement of blank hair, with two transitions: m/z 315.3 to m/z 259.2 (top) and 193.2 (bottom). B) Total ion current (TIC) and extracted ion chronograms (EIC) of THC and most abundant fragments with 5 ppm mass tolerance from DART-quadrupole orbitrap fragment ion scan of blank hair. C) Total ion current (TIC) and extracted ion chronograms (EIC) of THC and most abundant fragments with 1 Da window from DART-quadrupole orbitrap fragment ion scan of blank hair. D) Total ion current (TIC) and extracted ion chronograms (EIC) of THC and most abundant fragments with 50 ppm mass tolerance from DART-QTOF fragment ion scan of blank hair. E) Total ion current (TIC) and extracted ion chronograms (EIC) of THC and most abundant fragments with 1 Da window from DART-QTOF fragment ion scan of blank hair.*

Selectivity enhancement by use of TWIM

As shown in Figure 1D, the QTOF instrument had sufficient resolution when used in high resolution mode. However, the sensitivity was at least threefold lower compared to resolution mode on the same instrument. In resolution mode, similar DART hair scans were obtained as when using the triple quadrupole instrument: relatively abundant isobaric ions originating from the hair matrix hamper the differentiation between authentic THC signal and endogenous background ions signals. The use of TWIM to separate THC from the interfering isobaric ions in resolution mode was therefore investigated. Between *m/z* 315.15 and 315.35, THC and three, non-baseline separated, isobaric ions were detected (Figure 3A). From the ion mobility data, it was concluded that these ions had different drift times and differentiation was thus possible. When only the THC peak was selected based on both its exact mass and drift time, the unwanted signal originating from blank hair could be reduced three times (Figure 3B, inserts). In the resulting hair scans, it was easier to distinguish authentic THC signal from noise and only minor sensitivity loss was observed when TWIM was used, preserving the higher sensitivity compared to the use of high resolution mode. The higher selectivity by use of TWIM made the QTOF instrument more suitable for DART hair scanning. However, still some background signal from blank hair matrix was observed, as in the triple quadrupole measurements. Compared to orbitrap instruments, where no unwanted overlap with isobaric ions and thus no noise was detected, the QTOF instrument was less selective even when TWIM was used.

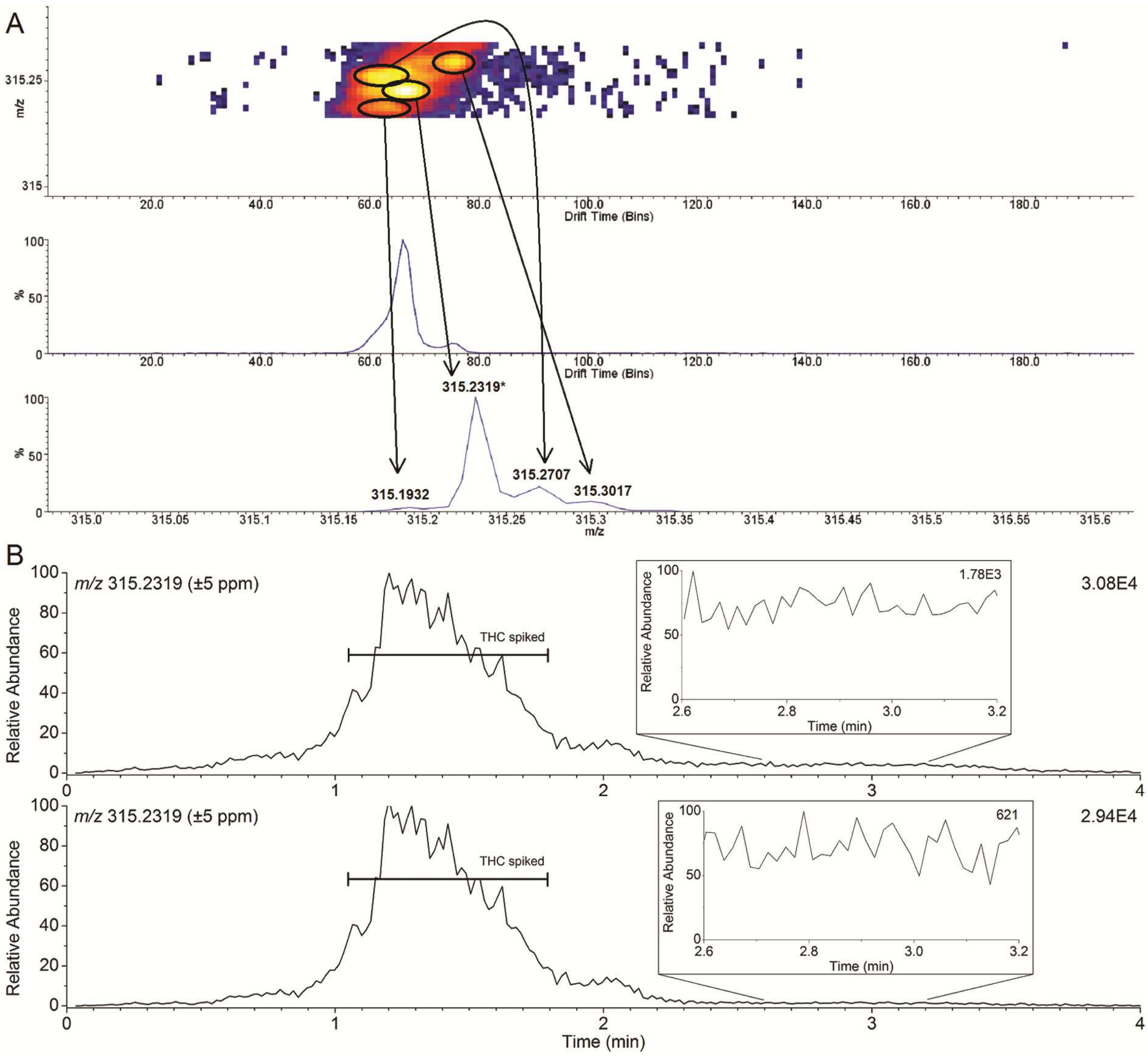

Figure 3. *A) TWIM data of DART-TOF analysis of secondary THC standard spiked on blank hair. Top: m/z vs. drift time; middle: reconstructed ion drift time distributions; bottom: MS spectrum, the $[M+H]^+$ ion of THC is indicated by an asterisk. B) Extracted ion chronogram (EIC) of DART-QTOF analysis of secondary THC standard spiked on blank hair based on m/z 315.2319 (top) and both m/z 315.2319 and drift time (bottom) with magnified regions of non-spiked hair.*

DART analysis of cannabis user hair samples

The conclusions regarding the selectivity of the different mass spectrometers led to a final comparison of the performance of the orbitrap and QTOF instruments for DART analysis of authentic cannabis user hair samples. As discussed in the previous sections, the selectivity of orbitrap instruments is sufficient because of their high resolution and the use of orbitrap instruments for the analysis of cannabis user hair has already been reported.[20,21] Full-scan high-resolution data, in combination with data-dependent product ion scans, was used for structural confirmation of the presence of THC and other drugs of abuse in drug user hair samples by exact mass and product ion ratios.[21] Figure 4A shows the extracted ion chronograms (EIC) of a drug user hair sample using the QTOF instrument in resolution mode, with and without selection of the drift time of THC. Only a minor drop in signal was observed when selecting the drift time of THC, however, the resulting mass spectrum is much cleaner compared to the mass spectrum when no drift time is selected (Figure 4B). The results shown in Figure 4 show that TWIM can be used to reduce the risk of THC overlapping with isobaric compounds, and thus reduce the risk of false-positive results. Nevertheless, after selection of the drift time of THC in a blank hair scan, still some background signal was detected at the *m/z* of THC (Figure S4). This is caused by the insufficient resolution of the TOF mass analyzer, indicating that not all isobaric ions with an exact mass very close to the mass of THC are filtered out by drift time selection. The same issue as described above for the triple quadrupole measurements occurs, hampering differentiation between blank hair and positive hair.

In forensic hair analysis, cut-off values can be used to identify chronic drug use,[30] and thus a detection limit below this value is needed to make the DART hair scan method suitable for use in forensic applications. The DART hair scan method using orbitrap instruments showed to have the necessary sensitivity for the analysis of cocaine in hair samples,[21] but the detection limit of THC[20] did not comply with the cut-off value recommended by the Society of Hair Testing (SoHT).[30] In this research, sufficient selectivity could only be obtained using orbitrap instruments. Unfortunately, the sensitivity of the present generation (quadrupole) orbitrap instruments could, as previously found, only be used to detect heavy cannabis use and was not sufficient to analyze THC in hair samples below the cut-off value recommended by the SoHT.[30] To analyze hair samples with low THC concentration, the previously described LC–MS/MS method after extensive sample clean-up, with a limit of detection below the cut-off value, should still be used.[20,27]

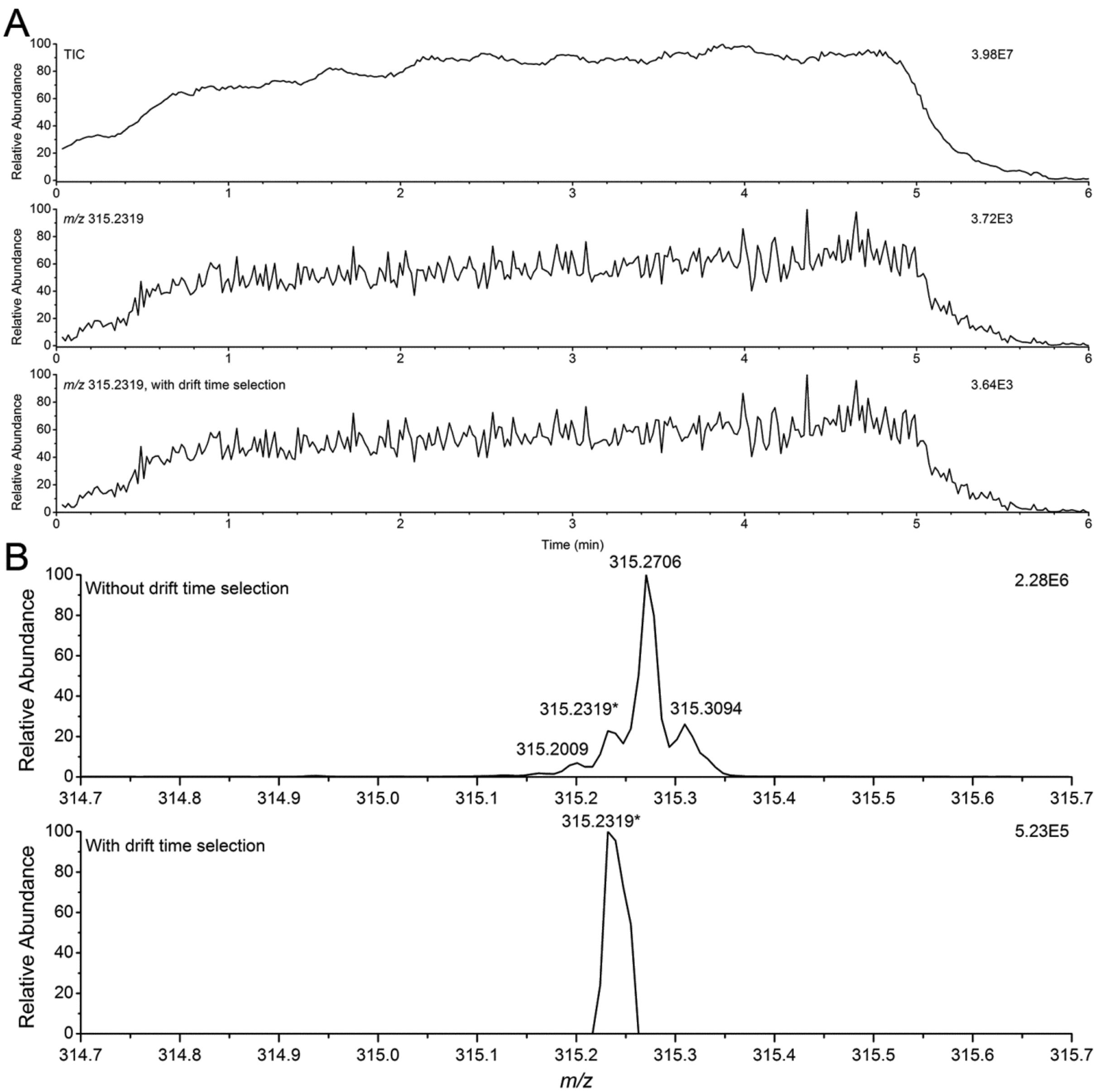

Figure 4. *A) Total ion current (TIC) and extracted ion chronograms (EIC) of DART-QTOF analysis of a cannabis user hair sample. Top: TIC; middle: EIC based on m/z 315.2319; bottom: EIC based on both m/z 315.2319 and drift time (bottom). B) DART-QTOF MS scans in resolution mode of a cannabis user hair sample. Top: no drift time selection; bottom: with drift time selection. The intact $[M+H]^+$ ion of THC is indicated by an asterisk.*

Conclusions

Selectivity and sensitivity of different mass spectrometers for the DART analysis of intact cannabis user hair samples were investigated. A major gain in sensitivity could be achieved using a triple quadrupole instrument in MRM mode, however, the nominal mass resolution did not provide enough selectivity resulting in a high background signal from the blank hair matrix in accordance with a theoretical assessment of the selectivity of the ion transitions used. Several isobaric ions with the same nominal mass as THC were detected using high resolution instruments, showing that DART-MS hair analysis with insufficient resolution could lead to false-positive results. Since endogenous isobaric ions were also observed during other applications of direct and ambient MS such as the detection of cocaine from drug user hair[21] and control of anisatin in star anise[24,31], the risk of false-positive results in direct and ambient MS is not limited to the detection of THC as investigated in this study. To differentiate between THC and the isobaric ions originating from the hair matrix, a resolution of at least 30,000 FWHM is needed. From the mass spectrometers tested, the (quadrupole) orbitrap instruments and QTOF instrument in high resolution mode had sufficient resolution, while the quadrupole orbitrap instrument had at least a three times higher sensitivity compared to the QTOF and orbitrap instruments. Using TWIM in the QTOF instrument, THC and the other isobaric ions could be separated based on their drift time. By this reduction of the background originating from isobaric ions, selectivity could be gained for the QTOF instrument in the more sensitive resolution mode. However, the background signal could not be totally reduced, thus hindering the differentiation between background signal originating from endogenous compounds and THC from drug use in user hair samples. Since unwanted overlap of THC with isobaric ions did not occur utilizing the high resolution of the (quadrupole) orbitrap instruments, and thus no noise was detected when analyzing blank hair samples, these instruments were found to have the highest selectivity for this purpose and are consequently the instrument of choice for DART hair scanning of drug user hair samples. The use of other mass spectrometers with high mass resolution, such as a Fourier transform mass spectrometer, could be an alternative but was not considered in this study.

The results presented in this research show that care has to be taken during direct or ambient MS analysis of hair samples, including mass spectrometry imaging of single hairs, due to possible overlap of endogenous isobaric ions with the compounds of interest. High resolution mass analyzers should be used to avoid false-positive result. It should be noted, however, that DART, as used in this research, is already a relatively clean ionization technique compared to, for example, MALDI and DESI. Therefore, it can be expected that other direct ionization methods will suffer even more from possible interfering isobaric ions. We recommend the forensic community to accept direct ionization and mass spectrometry imaging methods provided that fit-for-purpose selectivity of the methods is convincingly demonstrated and validated.

Acknowledgments

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Supporting information Chapter 5

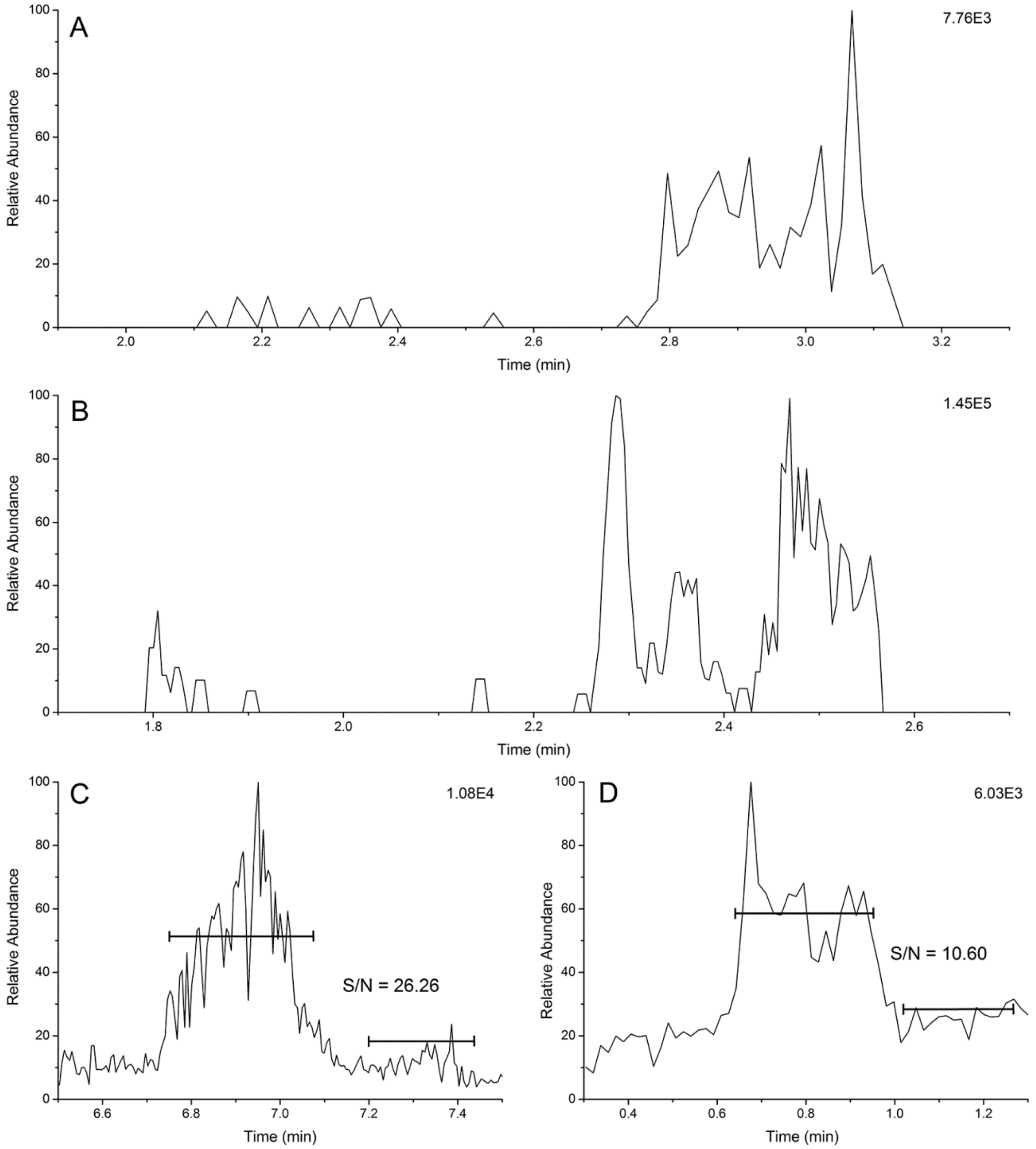


Figure S1. *A) Extracted ion chronogram (EIC) of THC, m/z 315.2319, in a DART-orbitrap scan of 0.16 and 0.47 ng THC showing an interrupted peak and a continuous peak. The continuous peak is designated as the lowest detectable concentration. B) EIC of THC, m/z 315.2319, in a DART-quadrupole orbitrap scan of 0.026 and 0.052 ng THC. C) Triple quadrupole chronogram with signal-to-noise ratio of the analysis of 0.047 ng THC on mesh. D) QTOF chronogram with signal-to-noise ratio of the analysis of 0.16 ng THC on mesh.*

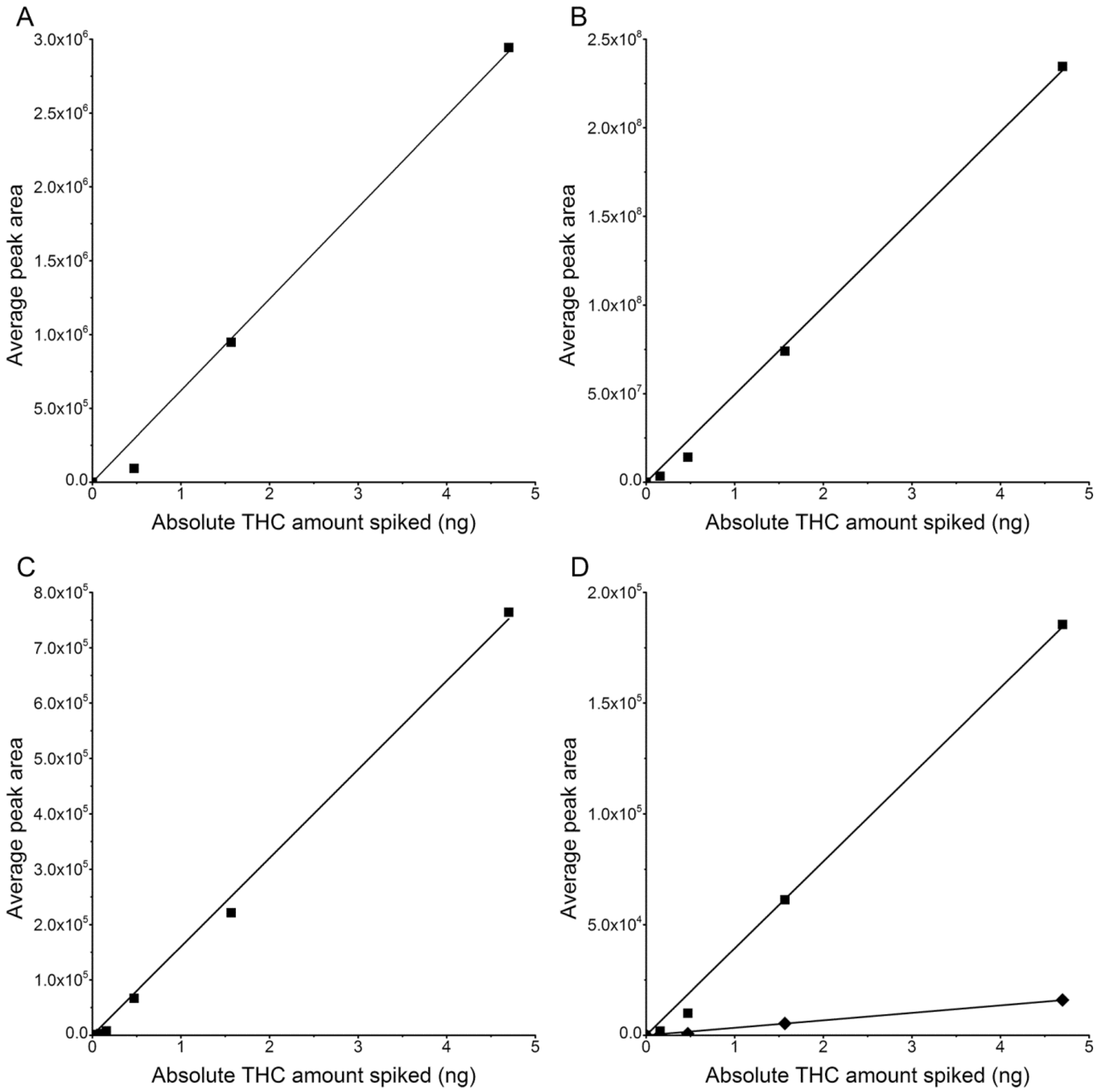


Figure S2. *Calibration curves of different secondary THC standard dilutions analyzed using different mass spectrometers: A) orbitrap instrument, B) quadrupole orbitrap instrument, C) triple quadrupole instrument, and D) QTOF instrument in resolution (square) and high resolution mode (diamond).*

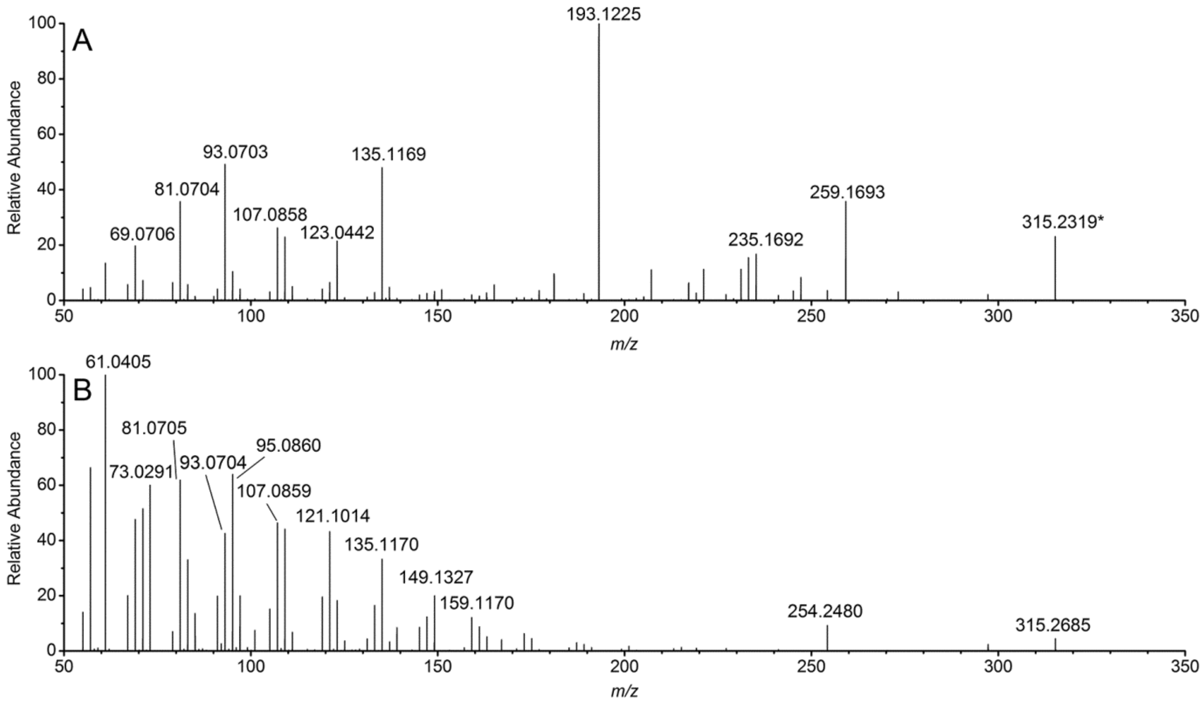


Figure S3. *DART quadrupole orbitrap MS/HRMS product ion spectra (normalized collision energy (NCE)=25), obtained by analysis of A) blank hair spiked with secondary THC standard, and B) blank hair. The intact $[M+H]^+$ ion of THC is indicated by an asterisk.*

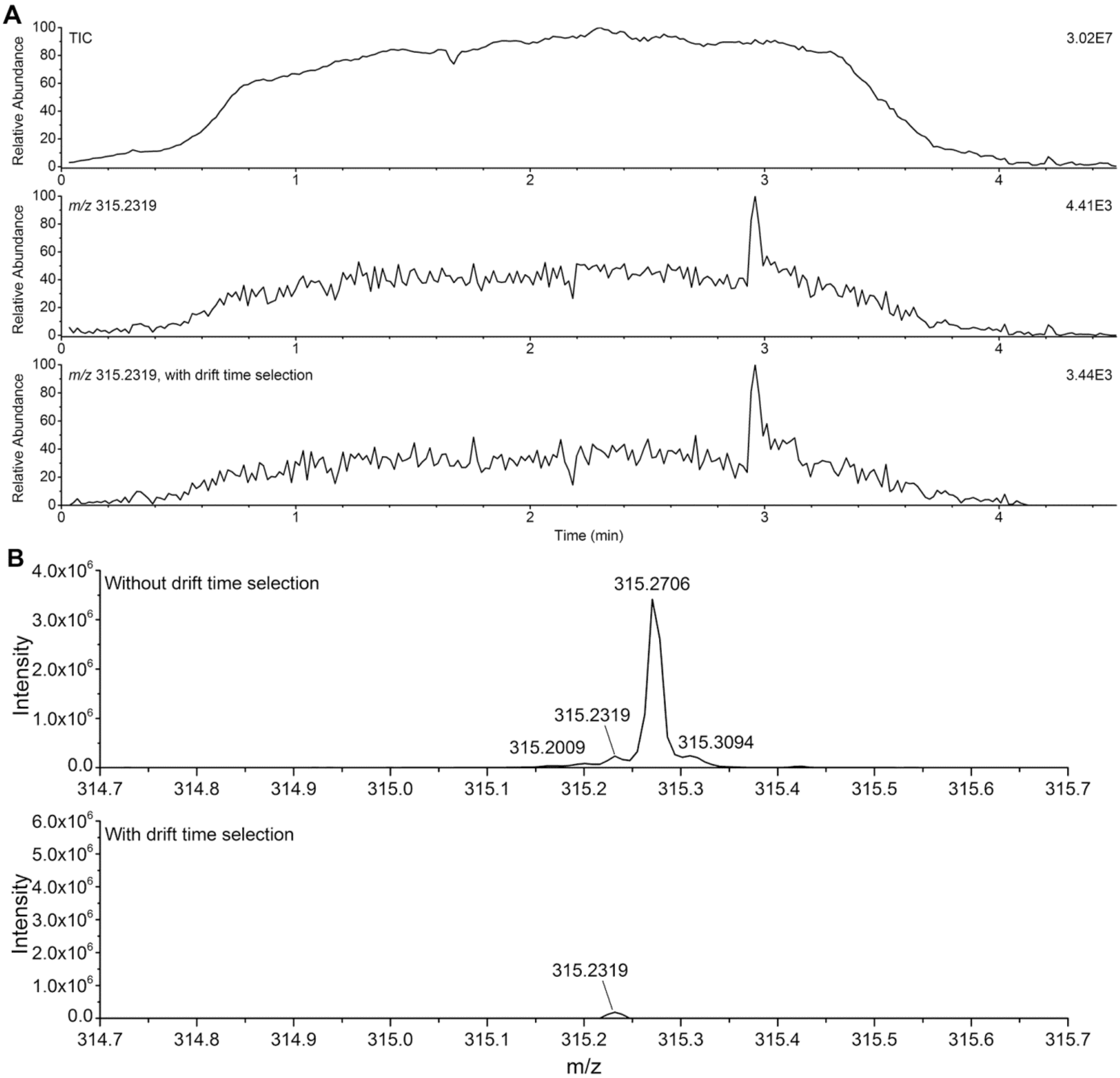
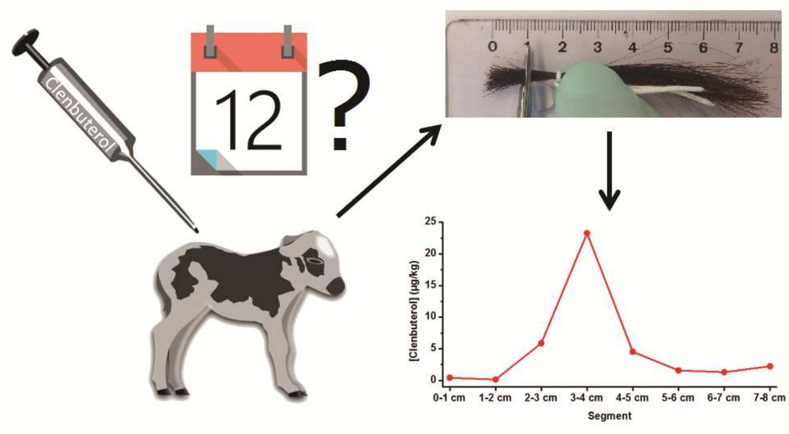


Figure S4. *A) Total ion current (TIC) and extracted ion chronograms (EIC) of DART-QTOF MS analysis of blank hair. Top: TIC; middle: EIC based on m/z 315.2319; bottom: EIC based on both m/z 315.2319 and drift time (bottom). B) DART-QTOF MS spectrum in resolution mode of blank hair. Top: no drift time selection; bottom: with drift time selection.*

Chapter 6

Ultratrace LC–MS/MS Analysis of Segmented Calf Hair for Retrospective Assessment of Time of Clenbuterol Administration in Agriforensics



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Abstract

In agriforensics, time of administration is often debated when illegal drug residues, such as clenbuterol, are found in frequently traded cattle. In this proof-of-concept work, the feasibility of obtaining retrospective timeline information from segmented calf tail hair analyses has been studied. First, an ultraperformance liquid chromatography−tandem mass spectrometry (UPLC–MS/MS) hair analysis method was adapted to accommodate smaller sample sizes and in-house validated. Then, longitudinal 1 cm segments of calf tail hair were analyzed to obtain clenbuterol concentration profiles. The profiles found were in good agreement with calculated, theoretical positions of the clenbuterol residues along the hair. Following assessment of the average growth rate of calf tail hair, time of clenbuterol administration could be retrospectively determined from segmented hair analysis data. The data from the initial animal treatment study (n=2) suggest that time of treatment can be retrospectively estimated with an error of 3−17 days.

Introduction

Clenbuterol is a β-agonist approved for therapeutic use in some countries. However, it can also be used illegally, as a growth promoter for cattle. It increases the muscle-to-fat ratio and stimulates overall animal growth.[1-3] Clenbuterol-contaminated meat can cause food intoxication with severe consequences such as heart disorders,[4,5] and it has therefore been banned for use as a growth promoter in cattle in the European Union since 1988.[6] Many different methods for the analysis of clenbuterol and other β-agonist residues in different matrices have been published over the years.[1,2,7-23] Among these methods are approaches based on immunoassays,[7] receptor assays,[24] or biosensors,[1,8,9,21] but these methods lack either sensitivity or selectivity. To overcome these problems, gas chromatography−mass spectrometry (MS) and liquid chromatography (LC)−tandem MS (MS/MS) have been used to analyze a large range of β-agonists.[2,10-16,18-20,22,23] One of the difficulties that occurs when residues of a banned substance such as clenbuterol are found in animals, however, is the fact that animals are frequently traded. The owner can thus claim that a previous owner was responsible for the illegal administration of the detected substances. Current analysis methods are not sensitive enough to be used for estimated time of drug administration. To achieve this, human hair forensic techniques may be equally applicable in veterinary control and agriforensics.

Hair is a very interesting sample matrix because many compounds accumulate in hair via the bloodstream. Hair pigmentation can have an influence on the incorporation of compounds such as clenbuterol into the hair. Gleixner *et al*. found that more clenbuterol was accumulated into black hair compared to hair with less pigmentation; however, clenbuterol was detectable in hair samples regardless of the level of pigmentation.[25] The prolonged detectability of drugs in hair versus blood or urine makes it a powerful option for forensic investigations.[26,27] Because the average hair growth rate of human head hair is known, analysis of longitudinal segments can be applied to achieve a retrospective timeline of drug intake.[28,29] Longitudinal hair analysis enables correlation of, for example, drug-facilitated crime with the time of drug administration.[28] Typically, 1−3 cm longitudinal hair segments are used for drug analysis, corresponding to multiple months of hair growth.[30,31] Over the past 10 years, several attempts have been made to apply segmented hair analysis to animals, but primarily for horses.[32-40] Dunnett *et al*. published several papers describing segmented analysis of different drugs in horse hair, mainly focused on antibiotic residues. The distributions among tail hairs were consistent with the time of drug administration, although the used segment sizes (2.5−5 cm) were somewhat large compared to human forensic applications.[32-35] Similar approaches on horse hair have been reported by Anielski *et al*.[36] and Gray *et al*.[39] for the detection of anabolic steroids and related compounds. Furthermore, segmented hair analysis has also been used to retrospectively determine stress factors in the hair of bears[38] and orangutans.[40] The smallest segment length used in previous animal studies is 2 cm,[32-36,38-40] which does not allow a very accurate estimation of time of drug administration. Until now, segmented hair analysis has not been applied to bovine (calf) hair or to the illegal use of β-agonists such as clenbuterol.

In the current, preliminary, study, hair segments of approximately 10 mm were used to enable a more accurate retrospective timeline assessment, preferably with a margin of error on the order of weeks of hair growth (instead of months), which can be helpful in court cases when the time of illegal drug administration is critical in relation to animal ownership. For reliable retrospective timeline assessment, hair must be sampled following the forensic hair sampling protocol, as recommended by the Society of Hair Testing.[31] Sampling of a pencil-thick lock of hair by cutting as close to the skin as possible, in combination with the smaller segment sizes, results in much smaller amounts of hair, on the order of 25 mg instead of 50−1000 mg of hair, being available for analysis. Therefore, an ultratrace analysis method with a very low limit of detection was needed to be able to detect the very low residue levels in the much smaller samples. Apart from that, the average growth rate of bovine hair needed to be assessed, to convert the results of the segmented hair analysis to a retrospective timeline of drug administration. To this end, the analytical method of Nielen *et al*.[18] was adapted to achieve ultratrace detection of clenbuterol in segmented hair samples. Locks of bovine tail hair, obtained from a controlled treatment (n=2) study, have been used to test the feasibility of the method and to obtain profiles of the clenbuterol distribution along locks of calf tail hair.

Materials and Methods

Chemicals and materials. All chemicals used were of (at least) analytical grade. Acetone, acetonitrile, ethyl acetate, and methanol were purchased from Actu-All Chemicals (Oss, The Netherlands). Acetic acid, ammonia, formic acid, hydrochloric acid, sodium acetate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Chloroform was purchased from BioSolve (Valkenswaard, The Netherlands). Purified water was from a Milli-Q system (Millipore, Bedford, MA, USA). Clenbuterol hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA), and clenbuterol-d_6 internal standard was custom-synthesized previously at Wageningen University and Research Centre (Wageningen, The Netherlands).

Hair samples. Blank hair samples were collected from the backs of different calves by shaving and were used for method development only. Incurred hair samples were collected from the tail of two male black-and-white Holstein Friesian calves of around 100 kg in a controlled treatment study performed at Ghent University (Ghent, Belgium). For ethical reasons, only two calves were dosed with clenbuterol, a banned substance, in this initial study. The calves were held in a separate stable, away from other cattle. The tails of the calves consisted of white hair only. After a 12 day acclimatization period, hair from the distal end of the tail of the two calves was sampled to obtain additional blank hair samples. Following the acclimatization period, the calves were treated for 14 days with clenbuterol. This animal study was approved by the Ethical Commission of Ghent University, under approval code EC2012/013, and took place from April until November. The treatment consisted of an oral dose of 1.2 µg clenbuterol (expressed as free base)/kg body weight dissolved in milk, once in the morning and once in the afternoon. Next, tail hair from the calves was sampled approximately once a month, for half a year. The detailed treatment and sampling schedule is shown in Figure 1A. At each sampling occasion, at least three pencil-thick hair samples were collected from the distal end of the tail of the calves, using the forensic hair sampling protocol recommended by the Society of Hair Testing.[31] Repeated sampling of the tail of the calves over the duration of the study caused differences in length of locks sampled on the same day, because some parts of the tail contained only shorter, newly grown, hairs due to previous sampling in that area. The resulting hair samples had a weight of approximately 30 mg/cm. For segmented analysis, hair samples were divided into 1 cm segments, starting from the proximal end.

Assessment of hair growth rate. The average growth rate of calf tail hair was determined both for continued growth of existing hairs (length at start = 4.1 ± 0.5 cm), as well as for newly grown hair. Six female calves (black-and-white Holstein Friesian) were used, 1−3 weeks old (around 50 kg) at the start of the study. Continued hair growth was monitored by measuring the hair length of three locks of clearly marked tail hair during 8 weeks. To determine the average growth rate of newly grown hair, three other locks of tail hair from the same calves were cut as close to the skin as possible at the start of the study. The length of newly grown hair was also monitored during 8 weeks (one data point per week). From these results, average tail hair growth rates were determined per calf, which were then used to calculate the overall growth rate and standard deviation for the six calves. Finally, the average of the data from the first 26 days was used for all calculations regarding the theoretical position of the clenbuterol residues (see Results and Discussion).

Sample preparation. The sample preparation of the hair samples was adopted from that of Nielen *et al*.[18] and adjusted to much smaller sample sizes. In short, 25 mg of hair were cut into small pieces (<3 mm), and 5.0 µL of internal standard was added (10 µg clenbuterol-d_6/liter). One milliliter of 1.0 M sodium hydroxide was added, and the samples were heated for 2 h in a water bath at 65 °C. After the samples had cooled to room temperature, 1.0 mL of 1.0 M hydrochloric acid and 1.25 mL of 0.25 M sodium acetate buffer (pH 4.8) were added. Next, the samples were centrifuged for 10 min at 2000*g,* and the supernatant was transferred into a clean test tube. Prior to an additional centrifugation step, the pH of the supernatant was checked and, if necessary, adjusted to 4.8. The resulting supernatant was subjected to mixed-phase solid phase extraction (SPE) cleanup as described.[18] In short, the column was conditioned with 3 mL of methanol and 3 mL of sodium acetate buffer before the sample was introduced. After sequential washings with 1 mL of 1.0 M acetic acid, 6 mL of methanol, and 2 mL of acetone/chloroform (1:1), the retained compounds were eluted using 7.5 mL of 1% ammonia in ethyl acetate. After evaporation to dryness under a N_2 flow at 40 °C, the residue was reconstituted in 100.0 µL of 0.24% formic acid, and a 10.0 µL aliquot was injected in the LC–MS/MS system.

LC–MS/MS method. The ultrahigh-performance liquid chromatography (UHPLC)–MS/MS analysis was carried out as follows. An Acquity UPLC system (Waters, Milford, MA, USA) was coupled to a Xevo TQ-S MS system (Waters), equipped with an electrospray ion source. The MS was used in positive ionization mode using the following conditions: capillary voltage, 3.0 kV; cone voltage, 20 and 25 V for clenbuterol and clenbuterol-d_6, respectively; source temperature, 150 °C; desolvation temperature, 400 °C; collision energy, 30 and 15 eV for clenbuterol and clenbuterol-d_6, respectively. Clenbuterol and clenbuterol-d_6 were analyzed in multiple reaction monitoring (MRM) mode using the following ion transitions: *m/z* 277.2 to *m/z* 132.1, 168.1, and 203.2 for clenbuterol and *m/z* 283.2 to *m/z* 204.1 for clenbuterol-d_6, respectively. Separation was achieved by using an Acquity BEH-C18 column (2.1 × 100 mm, 1.7 µm; Waters) at 40 °C and a flow rate of 0.4 mL/min. The mobile phases consisted of 25 mM formic acid in water (mobile phase A) and in acetonitrile (mobile phase B). The used gradient was as follows: isocratic elution for 1 min at 5% B, followed by a gradient applied to 35% B in 8.5 min and then a steep gradient toward 100% B in 0.3 min. After 2.2 min at this condition, the system was re-equilibrated for 3 min at 5% B. Data analysis was performed using MassLynx V4.1 (Waters). Matrix-matched standards (MMS) were used for clenbuterol quantification in the hair samples. In short, clenbuterol standard was added to blank calf hair in concentrations ranging from 0.5 to 10 µg clenbuterol (expressed as free base)/kg hair. The resulting calibration curve was then used for concentration calculations in the hair samples. Throughout this work, all clenbuterol is expressed as the free-base basis.

Method validation. The linearity of the MMS calibration curve was determined by analyzing the following clenbuterol concentrations in duplicate: 12.5, 25, 50, 100, and 250 pg per 25 mg of hair. The recovery was evaluated by spiking three blank hair samples with 50 pg clenbuterol/25 mg of hair. After sample pretreatment, 5.0 µL of internal standard was added just before injection instead of during spiking. Both experiments were executed on three different days. Further validation of the method regarding within-day repeatability, within-laboratory reproducibility, accuracy, decision limit (CCα), and detection capability (CCβ)[41] was performed using blank hair samples spiked at three concentration levels: 25, 50, and 100 pg/25 mg of hair. A total set of 18 replicates for the 25 and 100 pg/25 mg concentration level and 21 replicates for the 50 pg per 25 mg concentration level were analyzed on three different days. In addition, 21 blank hair samples were analyzed with the addition of internal standard only.

Data and statistical analysis. The hair growth data presented are mean values with corresponding relative standard deviations (RSD). Student's *t* test (two-sample assuming unequal variances) was used to assess significance of the difference between hair growth rates ($P < 0.01$). All validation data were evaluated using ANOVA. The analytical reproducibility of the method (10.6%) is shown as error bars in Figures 2−4.

Results and Discussion

Ultratrace LC–MS/MS method development and validation

The improved LC–MS/MS method for clenbuterol in hair allowed the analysis of hair samples as small as 25 mg, 2−40x lower than in previously reported methods.[1,2,10,19,21,22] The within-day repeatability and within-laboratory reproducibility RSD were <9.6 and <10.6%, respectively, at all spike levels. Accuracy was 92−101% with an average recovery of 37%, which was slightly lower than the average recovery (49%) of the method by Nielen *et al*.[18]

The incomplete recovery is possibly caused by incomplete reconstitution in only 100 µL, which volume is significantly lower than the 300 µL used by Nielen *et al.*[18] However, the lower reconstitution volume was necessary to obtain a final clenbuterol concentration high enough for LC–MS/MS analysis. Moreover, the added internal standard was capable of reproducibly correcting for the incomplete recovery, as shown by the accuracy and reproducibility data given above. Linearity of the MMS calibration curve was adequate in a range from 12.5 to 250 pg/25 mg hair (R^2 > 0.993). The resulting CCα and CCβ were 16.8 and 33.7 pg/25 mg hair, respectively. Compared to the CCβ reported in the previously described method[18] (<2500 pg/500 mg hair), we achieved a tremendous increase in absolute sensitivity, which was essential for the small sample amounts expected in this study. This sensitivity is similar to or better than that previously reported for clenbuterol in hair.[1,2,10,19,21,22]

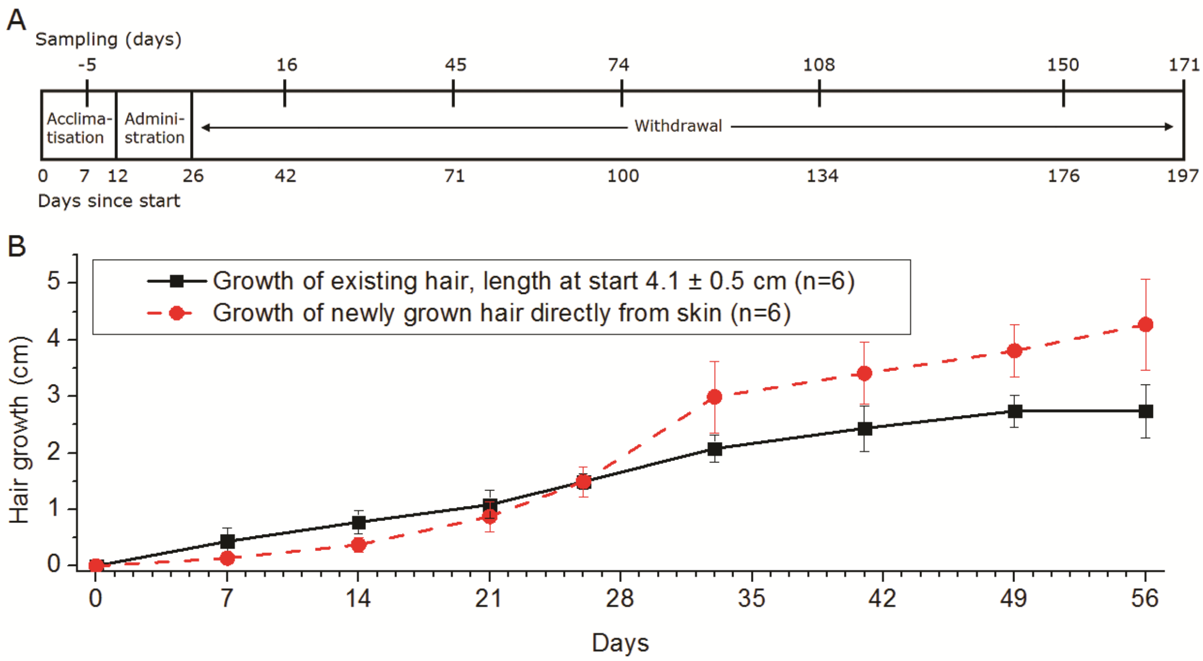

Figure 1. *A) Controlled animal treatment and sampling schedules. B) Average hair growth rate of calf tail hair, both as continued growth from an existing lock and as new growth (after cutting as close to the skin as possible). Error bars represent intra-animal RSD (n = 6).*

Average tail hair growth rate

The average tail hair growth rate for calves has been determined for both continued growth on existing hairs and newly grown hair. For both types of hair, the locations were similar as used for sampling in the controlled treatment study. The length of three locks of tail hair and the length of newly grown hair on three other spots, after cutting as close to the skin as possible, were recorded over a period of 8 weeks. The resulting hair growth curves are shown in Figure 1. During the first 26 days, the growth rate was equal ($P < 0.01$) for both existing and new hair: 0.57 mm/day (± 0.05 and ± 0.10 mm/day for, respectively, continued and newly grown hair). After the first 26 days, the growth rate of existing hair plateaued, resulting in an average growth rate over 56 days of 0.49 ± 0.08 mm/day. During the 49 and 56 day time points, some of the locks became shorter or stayed the same length, indicating that the decreasing average growth rate was most likely caused by the breaking off of brittle tips of longer hairs. The latter hypothesis has been confirmed by dyeing tips of long tail hairs. After 2 weeks, the dyed part of the hair became shorter and new hair was formed at the proximal end of the hair, indicating that hair continued to grow, even though the total length did not increase. Hair zones containing drug residues will keep growing toward the distal end of the hair. The implications of breaking off brittle tips on the final retrospective timeline assessment should be investigated in more detail in future studies. The average growth rate at 26 days, 0.57 ± 0.08 mm/day, has been selected for further calculations, because both types of tail hair showed similar growth at this time point and later data points already showed plateauing of the hair growth rate for existing hairs (Figure 1). Few reference data on the hair growth rate of calves are available; the only data published report 0.20−0.23 mm/day[42,43] but, unfortunately, these references do not provide experimental details, so it is unknown whether these reference data also relate to tail hair.

Segmented hair analysis for retrospective timeline assessment of clenbuterol administration

The sampled locks of calf tail hair were analyzed after cutting into 1 cm segments. In view of the average hair growth rate as described above, each segment corresponded to approximately 17.5 days of hair growth. The resulting clenbuterol concentration profiles along the hair locks, prior to and up to 108 days after clenbuterol administration, are shown in Figure 2. Samples taken 108, 150, and 171 days after the end of the clenbuterol administration period did not contain clenbuterol in any hair segment. No clenbuterol was detected in the samples taken 5 days prior to clenbuterol administration (Figure 2), indicating that the calves did not come into contact with clenbuterol prior to the controlled administration and verifying once again that blank hair yields negative results. For the first calf (Figure 2A), peak clenbuterol concentrations were found in the 0−1 cm hair segment of the day 16 sample, in the 1−2 and 2−3 cm segments of the day 45 samples, and in the 3−4 cm segment of the day 74 samples. The longitudinal hair profiles from the samples obtained from the second calf (Figure 2B) showed peak clenbuterol concentrations in the 1−2 cm hair segment of the day 45 samples and in the 2−3 and 3−4 cm segments of the day 74 samples.

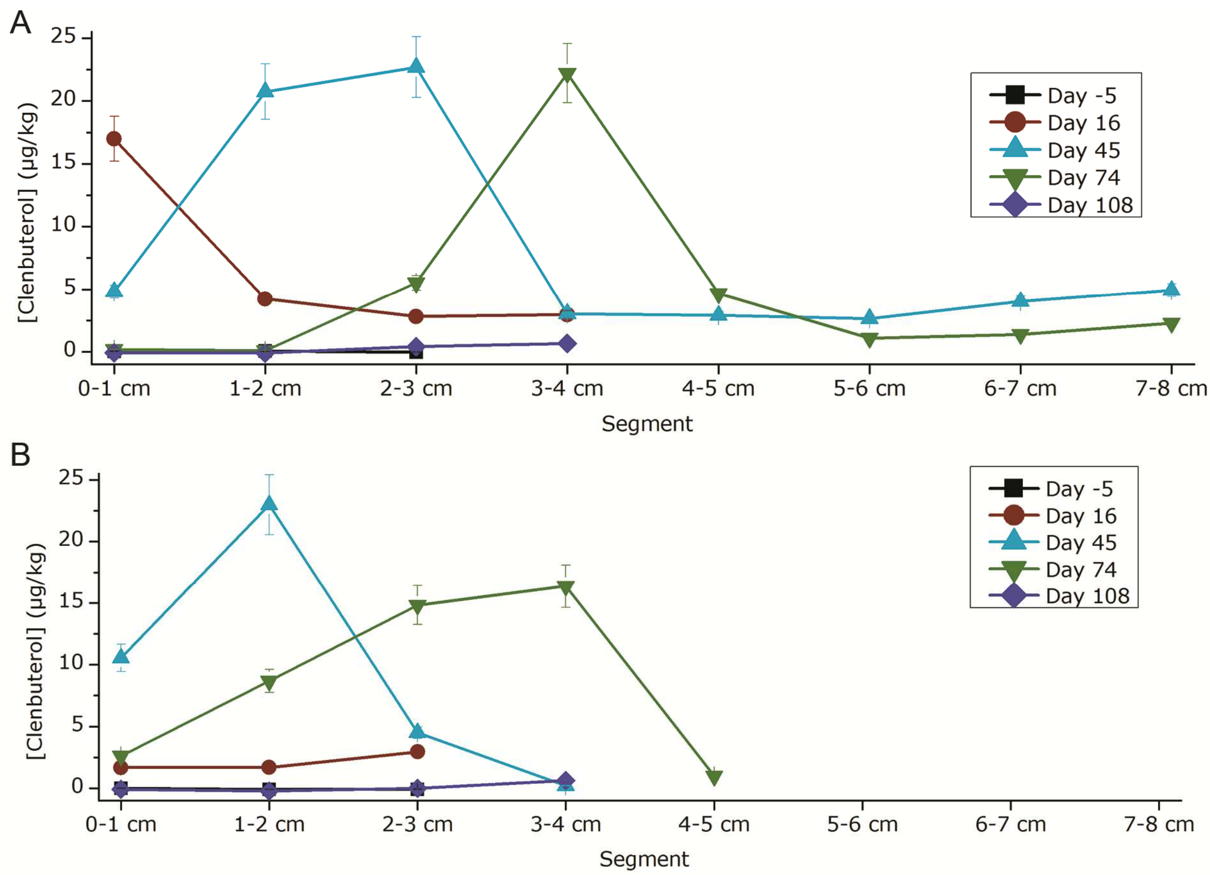

Figure 2. *Clenbuterol concentration profiles versus hair segments from calf tail hair samples, taken at different time points (days before or after the end of the clenbuterol administration period of 14 days), of two different calves (A and B). Error bars represent the analytical reproducibility of the method (10.6%).*

To establish the theoretical position of the clenbuterol residues in the hair lock samples, several issues have to be taken into account. First, clenbuterol was administered for 14 days (Figure 1A), theoretically resulting in incorporation into a longitudinal section of the hair of approximately 0.80 ± 0.11 cm, provided clenbuterol had been exclusively incorporated into hair via the hair root. The section in which clenbuterol was incorporated was, most likely, even larger because clenbuterol in cattle has a half-life time of 19 h during the distribution phase (first 3 days after administration) followed by a half-life time of 57 h during the elimination phase.[44] The majority (>95%) of the administered clenbuterol would have been metabolized or excreted from the body after 4 days. The additional days of unaltered clenbuterol in the blood circulation caused additional incorporation days in calf hair via the root. The prolonged presence of clenbuterol in the blood after withdrawal can add an extra 0.24 ± 0.03 cm to the length of the hair section containing clenbuterol. Furthermore, newly grown hair sections can be sampled only after clenbuterol reaches the skin surface. The skin thickness of calves is approximately 2.8 mm[42] and should be taken into account for the calculation of the theoretical position of the clenbuterol containing hair section. The theoretical positions of clenbuterol residues calculated using the average hair growth rate of 0.57 ± 0.08 mm/day and corrected for both the elimination time from the blood and the skin thickness are given in Table 1. The experimental data of hair segments containing clenbuterol peak concentrations were in good correlation with the calculated theoretical positions (Table 1). The theoretical position of the clenbuterol residues 108 days after administration would be around 5.5 cm, whereas the hair samples that could be taken at that time were of a maximum length of 4 cm. Thus, these 4 cm represent post-treatment hair only, in which clenbuterol could not have been incorporated. Besides the peak levels in the clenbuterol profiles along the hair segments, some segments showed some low residue levels of clenbuterol (e.g., day 16, 1−4 cm; day 45, 3−8 cm in Figure 2A). This is possibly caused by external contamination of hair via licking upon drinking the clenbuterol-containing milk[45] or by endogenous self-contamination via sweat, as described for human forensic practice by Henderson *et al*.[46] Another explanation for the low clenbuterol residue levels in segments not containing peak concentrations is axial diffusion of compounds along the hair, as described by Kintz *et al*.[47] However, both external contamination and axial diffusion will not interfere with the retrospective assessment of treatment time because in this preliminary trial only the position of the peak values in the clenbuterol concentration profiles along the hair segments was used. The peak values were much higher and could be easily distinguished from the slightly elevated levels caused by external contamination or axial diffusion. An initial attempt to remove external contamination by washing the hair segments with methanol prior to sample preparation did not reduce the detected traces of clenbuterol, in agreement with the findings of Hernández-Carrasquilla *et al*., who showed that methanol decontamination is insufficient to remove external β-agonist contamination from hair.[48] Because clenbuterol is a non-natural synthetic compound, residues in hair always indicate illegal use. Furthermore, the residue concentration following external contamination will be much lower compared to the peak concentration[45] and therefore not influence the assignment of the position of the clenbuterol peak along the hair.

In agriforensic practice, the time of drug administration will be unknown, and the segment of the hair in which the clenbuterol peak is detected must be converted to a timeline using an average hair growth rate. The skin thickness has been added to the start and end points of the segments, and the hair lengths have been converted to days of hair growth. The resulting retrospective estimations of time of clenbuterol treatment are included in Table 1. On basis of this initial animal trial (n=2), the retrospective estimations from segmented hair analysis were in good agreement with the actual period of clenbuterol administration. The accuracy of the retrospective estimation of the time of clenbuterol administration was 18 days when the clenbuterol peak concentration was detected in only one of the 1 cm long hair segments and approximately 35 days when detected equally in two subsequent hair segments (see, for example, the day 45 and 74 samples in panels A and B, respectively, of Figure 2). The experimental mean values for the retrospective time assessment show an error of only 3−17 days when compared to the actual time of clenbuterol administration (Table 1). It should be stressed that these results were obtained in a preliminary treatment (n=2) study. Nevertheless, the results suggest that segmented hair analysis can be successfully used in agriforensics to obtain a retrospective estimation of the time of clenbuterol administration to calves.

Table 1. *Theoretical Position of Clenbuterol Residues in Calf Tail Hair, Calculated Using an Average Hair Growth Rate of 0.57 ± 0.08 mm/day, Experimental Position from Segmented Tail Hair Analysis, and Retrospective Assessment of the Time of Drug Administration*

Actual sample collection time after treatment	Theor position of hair section containing clenbuterol residues (cm)	Exptl position of clenbuterol peak concn in segmented tail hair samples (cm)	Exptl retrospective time of clenbuterol admin (mean days ago and range)
day 16	0.28 ± 0.1 to 1.3 ± 0.2	0−1	13.5 4.8 ± 0.6 to 22.3 ± 3.0
day 45	1.9 ± 0.3 to 2.9 ± 0.4	1−3	39.7 22.3 ± 3.0 to 57.2 ± 7.7
day 74	3.5 ± 0.5 to 4.5 ± 0.6	2−4	57.2 39.7 ± 5.4 to 74.7 ± 10.1
day 108	5.5 ± 0.8 to 6.5 ± 0.9	not detected	not detected

Reproducibility of longitudinal clenbuterol profiles

To test the reproducibility of the profiles, subsamples taken from the same lock of hair and duplicate locks of hair, sampled at the same time, were analyzed. A lock of tail hair sampled 45 days after the end of the clenbuterol administration period was divided into two subsamples and 1 cm segments of these subsamples were analyzed. The conclusion with respect to the segments having the highest clenbuterol levels are comparable (Figure 3): both profiles show peak concentrations in the 1−2 cm segment, but for the second subsample, the peak clenbuterol concentration extended into the 2−3 cm segment. This may have been caused by uneven cutting of the hair due to bundling of the hair lock, which can result in slightly different sampling distances relative to the skin within a sampled lock. This phenomenon has been described in more detail by LeBeau *et al*.[49] and may result in two nonidentical subsamples after splitting of a lock of hair.

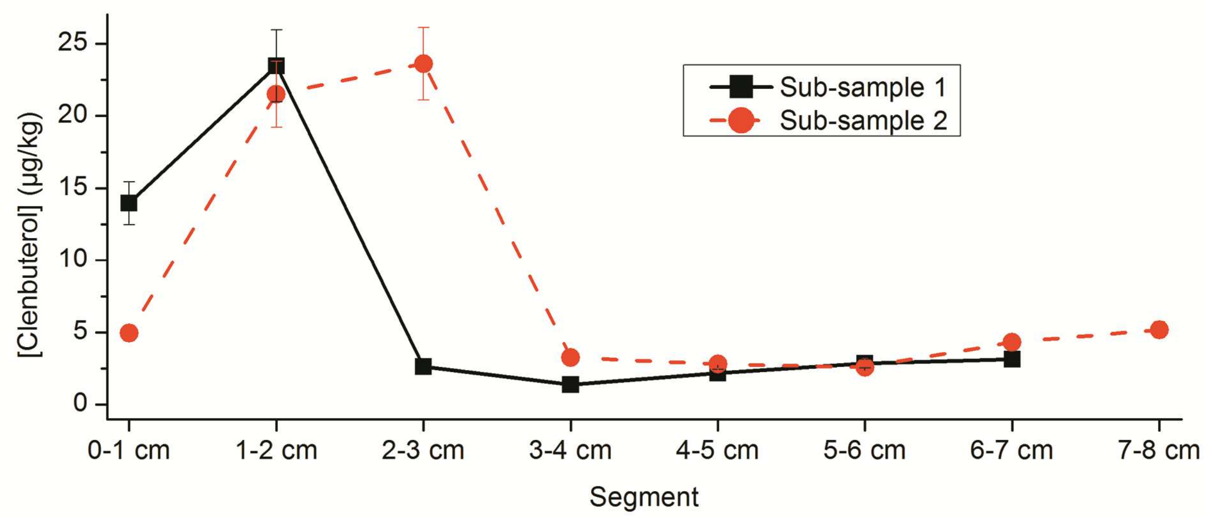

Figure 3. *Clenbuterol concentration profiles versus hair segments from calf tail hair. Subsamples were obtained from a lock of hair sampled 45 days after the end of the clenbuterol administration period. Error bars represent the analytical reproducibility of the method (10.6%).*

Figure 4 shows the concentration profiles of duplicate locks of hair, sampled at the same day. The duplicate samples, taken 45 days after administration (Figure 4A), show high clenbuterol concentrations in the first two segments and lower in the distal segments, which is consistent with the calculated position of the clenbuterol residues (Table 1). For the analyzed duplicate samples collected 74 days after administration, a lock of 3 cm and a lock of 10 cm were analyzed (see Figure 4B). [Note: the difference in length of these locks is most likely caused by the repeated sampling of the tail of the calves. Some parts of the tail contained only shorter newly grown hairs, because those areas had been sampled before, whereas other parts still contained longer hairs on areas that had not been sampled before (see Materials and Methods section).] An increase in clenbuterol concentration has been noted toward the 3−4 cm segment in both duplicate locks, and even though the length of the locks was not equal, the found trend is similar. A factor to be taken into account is the intra-animal variability: human hair forensics shows that the variation in residue concentrations between locks of hair from one person ranges from 14 to 62 %. This can cause differences between and within locks of hair as also observed in the present study (Figures 3 and 4). Note that the analytical variability of each data point is much lower (10.6%) according to the validation study. The position of the clenbuterol peak concentration, however, will not change by variation in the absolute concentration. In this respect, both samples showed comparable results: it can be stated that segmented hair analysis is reproducible in the assignment of the relevant segment position and, therefore, can result in a similar retrospective estimation of time of drug administration. It should be stressed, however, that inaccurate hair sampling can cause differences, even within a single lock of hair.[49] To reach the highest certainty about time of drug administration, multiple locks of hair should be sampled by cutting as close as possible to the skin and as straight as possible, in accordance with recommendations.[31]

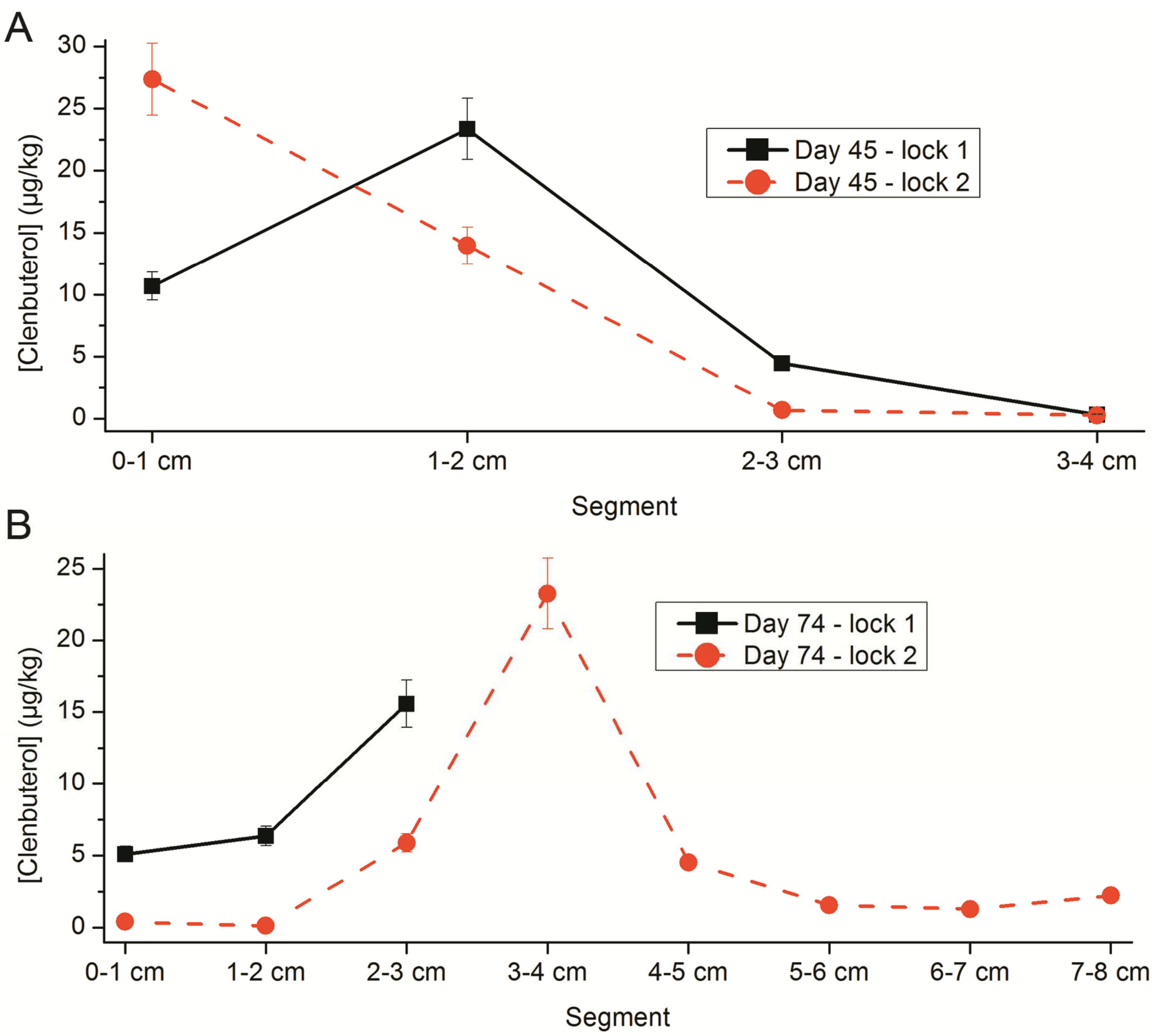

Figure 4. *Clenbuterol concentration profiles versus hair segments from duplicate locks of calf tail hair, sampled A) 45 and B) 74 days after clenbuterol administration. Error bars represent the analytical reproducibility of the method (10.6%).*

Future perspectives

The LC–MS/MS method developed showed a CCβ value of 33.7 pg for as little as 25 mg of hair, which represents a major improvement compared to the previously described method (CCβ <2500 pg for 500 mg of hair).[18] The resulting clenbuterol concentration profiles along the hair segments nicely shows the feasibility of retrospective estimation of the time of drug administration, with a mean error of 3−17 days, depending on the shape of the concentration profile of clenbuterol residues along the segments. To get a more precise retrospective estimation of the time of treatment, more reference profiles from different calves after controlled treatment should be analyzed and combined with more in-depth and longer lasting research toward differences in hair growth rates. In a follow-up study, both male and female calves and calves with different tail hair pigmentation should be included to gain more insight in the interanimal variation. Because many compounds accumulate in the hair of cattle, this method should be applicable to other banned substances as well, especially to other β-agonists. Extension of the scope can provide the agriforensics community with a powerful tool to estimate the time of illegal β-agonist administration. In many cases, an error on the order of a few weeks will be sufficient to support court cases in which (timelines of) illegal treatments are being challenged. Possibly, the retrospective timeline assessment could be improved further with trace analysis of even smaller segments. The latter may require entirely new analysis approaches such as longitudinal mass spectrometry imaging of intact hairs.[50]

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Chapter 7

General Discussion and Future Perspectives

General discussion

The use of hair analysis in forensic investigations often faces severe challenges. Hair evidence can, for example, be questioned due to the possibility of external contamination and distinguishing contamination from endogenously incorporated compounds can be difficult. The main goal of this thesis was to increase the value of hair evidence by critical evaluating current practices and the development of a novel hair analysis method, which enables fast acquisition and accurate retrospective timelines of drug exposure.

Critical evaluation of decontamination protocols

Even though external contamination is a major issue in forensic hair testing, no conclusive evaluation of decontamination protocols was available. As part of addressing the need for an evidence-based decontamination protocol, an extensive evaluation of wash strategies for the removal of cannabis contamination from hair samples is presented in **Chapter 2**. After wide-ranging tests of different solvents and aqueous solutions using single and multiple wash protocols, three protocols were found to remove almost all external cannabis contamination originating from different sources. Consideration of issues such as the environmental impact, labor input and time, as well as the recommendations of the SoHT, led to a new decontamination protocol for the removal of cannabis contamination consisting of a single methanol wash followed by a single aqueous SDS solution wash, followed by a Milli-Q water rinsing step. Decontamination is a delicate compromise between the removal of external contamination and preserving endogenously incorporated compounds, and therefore, a very important part of this study was the use of these protocols on drug user hair samples. With this evidence-based protocol, cannabis contamination can be removed, while the incorporated cannabinoids are not extracted. Because the incorporation of compounds into the hair matrix and the effectiveness of a wash protocol to remove compounds present as external contamination are very much dependent on the physicochemical properties of a substance, similar extensive studies will be needed to address the removal of contamination originating from other compound classes, such as cocaine, opioids and amphetamines.

While a recent study of cocaine decontamination protocols using MALDI and MetA-SIMS imaging suggested migration of external contamination, originating from rubbing cocaine salt powder on the hair or soaking of hair in cocaine solution, into the hair matrix during washing,[1] this phenomenon was not observed during the evaluation of the decontamination protocols for cannabinoid contamination originating from indirect contact with cannabis plant material or cannabis smoke. All hair samples were fully digested prior to LC–MS/MS analysis, releasing all cannabinoids from the hair matrix. Only insignificant amounts of the investigated cannabinoids were detected after using the selected decontamination protocols on intentionally contaminated blank hair samples, ruling out the possibility of cannabinoids migrating into the hair matrix during the decontamination process. The MetA-SIMS results in the cocaine decontamination study, however, were obtained using a mass spectrometer with rather low mass resolution. Insufficient mass resolution can lead to false-positive results as discussed in **Chapter 4** and **Chapter 5**, where it is shown that the required mass resolution to analyze cocaine using DART without interference from endogenous isobaric ions is at least 60,000 FWHM. Since no SIMS images of blank hairs were shown, it is unclear whether or not they yield false-positive results.

Another approach to distinguish between external contamination and incorporated compounds is briefly described in **Chapter 3**. Instead of decontamination by solvents, use of the newly developed DART hair scan method enabled to distinguish between external contamination and incorporated compounds through the differential release rate of external and internal THC at elevated temperatures. Initial tests of repeated DART scanning of hair samples showed promising results to determine the origin of detected THC. However, a more elaborate recent study with blank hair contaminated by several approaches and drug user hair samples indicated that repeated scanning could only be used to differentiate between blank hair spiked with methanolic cannabis extract using a TLC sprayer and all other samples tested (Figure 1A). No significant difference in rate of decrease of the THC signal was found between drug user hair samples and blank hair contaminated by cannabis smoke, indirect contact with cannabis plant material, or soaking in cannabis extract. The observation that hair samples can be repeatedly scanned suggests that DART analysis is not destructive. DART-scanned hair samples might thus still contain enough analytes for confirmatory testing using a more conventional analysis method.

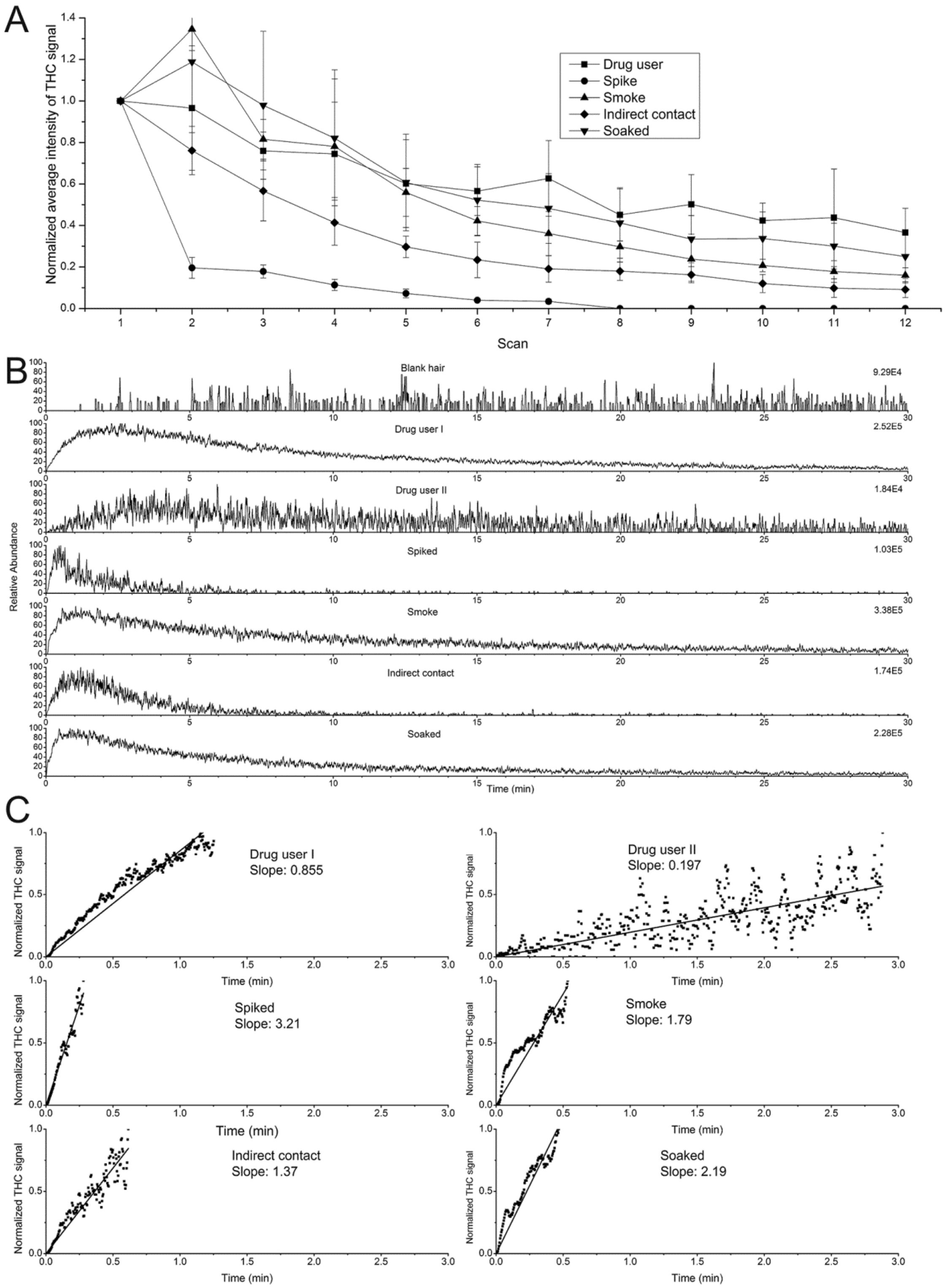

Figure 1. *A) Average intensity of THC signal in repeated longitudinal hair scans of drug user hair samples and blank hair samples contaminated using different approaches. B) EIC of THC (m/z 315.2319) of 30-minute static DART-HRMS measurements of blank hair, drug user hair samples and blank hair contaminated using different approaches. C) Normalized THC signal with slope from start of measurement to maximum THC signal.*

In addition to repeated DART scanning, a static DART measurement of one section of a hair sample during 30 minutes was investigated as a possible tool to differentiate between external contamination and incorporated compounds. SEM images of hairs after static DART measurement did not shown any substantial damage to the hair surface compared to untreated hair. The resulting patterns over time show some potential to distinguish between drug user hair samples and blank contaminated hair (Figure 1B). Based on the full pattern, again only blank hair spiked using a TLC sprayer behaves different from the other investigated samples: the THC signal drops rapidly after a short peak signal, while other analyzed samples show a slow decrease of the THC signal. When focusing on the first 5 minutes, however, it can be noted that the maximum THC signal is reached faster when contaminated hair samples are analyzed compared to drug user hair samples. The maximum THC signal for drug user hair samples was reached after 1−3 min, while the THC signal during the analysis of smoke, indirect contact, and soak-contaminated hair reached its maximum within 1 min (Figure 1B). Furthermore, the slope from start of the static measurement to the maximum was investigated (Figure 1C), showing a significant (P < 0.05) difference between drug user hair samples and contaminated blank hair samples. These initial results suggest that through static measurements, differentiation between THC originating from external contamination and incorporated THC is possible. This hypothesis should be more thoroughly tested. Patterns obtained from additional authentic drug user hair samples, different batches of contaminated blank hair, and deliberately contaminated drug user hair samples are needed before a final conclusion can be made regarding the applicability in forensic practice. Static DART measurement should furthermore be tested on contamination originating from other compound classes.

Supercritical fluid extraction (SFE) using carbon dioxide was briefly explored as another possibility to remove external THC contamination. THC and other cannabinoids are soluble in hexane and were therefore expected to be well soluble in supercritical carbon dioxide. High-level cannabis smoke-contaminated hair sub-samples were placed inside a stainless steel container and exposed to supercritical carbon dioxide while varying temperature, pressure and exposure time. For a comparison with conventional decontamination by wash steps, another sub-sample was decontaminated using the optimized protocol developed in Chapter 2. Afterwards, the samples were analyzed using the LC−MS/MS method described in Chapter 2 and 3. The results of this preliminary test are displayed in Figure 2A. Please note that, because an unrealistically high level of smoke contamination was used, a minor part of the THC contamination was not removed using the recommended wash protocol, in agreement with the results described in Chapter 2. Figure 2A shows that the amount of smoke contamination removed by supercritical carbon dioxide is at least comparable to that removed using the MeOH-SDS wash protocol. The results of this research, although preliminary, imply that SFE might be suitable as a decontamination method for THC on hair samples. Further testing on contaminated blank hair and authentic drug user hair samples is necessary; especially possible removal of incorporated compounds from drug user hair samples should be critically evaluated. Visual inspection of SFE-treated hair samples using SEM showed severe damage to some parts of the hair surface (Figure 2B), emphasizing the need to assess the impact of SFE on incorporated compounds. Accelerated solvent extraction (ASE) using elevated temperature and pressure might be considered as an alternative to the use of SFE.

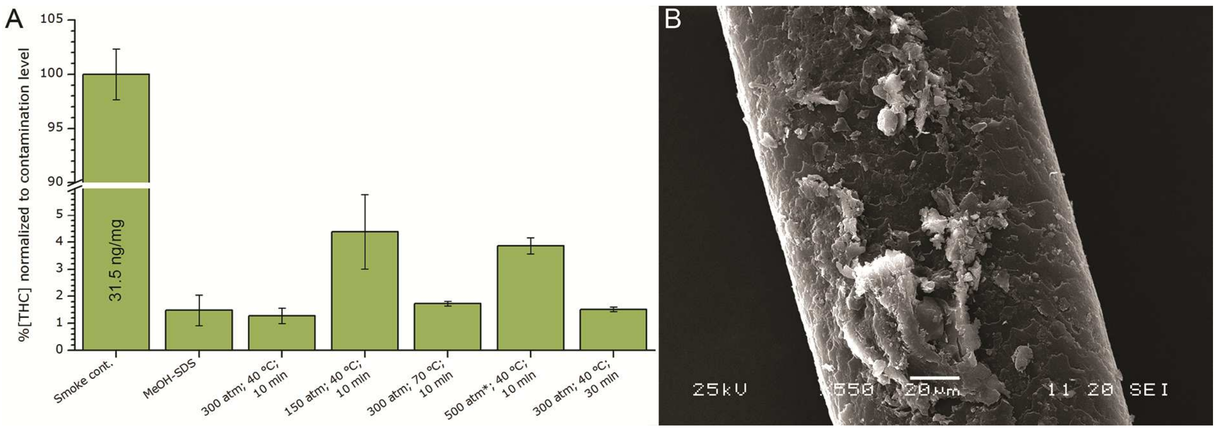

Figure 2. *A) Normalized THC levels in hair following wash and SFE decontamination after cannabis smoke contamination (n = 3). (* actual pressure during the dynamic part of the SFE cycle was approximately 380 atm) B) SEM image of hair after SFE treatment for 30 min at 300 atm and 40 °C using supercritical carbon dioxide.*

From this thesis and the above-discussed results, it can be concluded that not all contamination is alike. First of all, spiking of hair with a methanolic cannabis extract using a TLC sprayer results in contamination very different from that originating from more realistic sources such as smoke and indirect physical contact. This is most likely caused by the very small droplets of methanolic cannabis extract formed by the TLC sprayer, which upon reaching the hair will evaporate very rapidly and leave small concentrated spots of cannabinoids on the outer surface of the hairs exposed to the spray. Cannabis smoke and indirect contact with cannabis plant material will probably result in more widespread and adhering contamination due to, respectively, adsorption from the gas phase and rubbing of the contamination onto hair, explaining the difference in the results shown in Figure 1. Secondly, many published studies assessing contamination and/or decontamination mimic contaminated hair by soaking hair in analyte solutions.[1-7] As discussed in Chapter 2, contamination by soaking is unrealistic and more radical compared to smoke and indirect contact contamination, especially when high contamination levels are used, and the resulting contamination will be harder to remove. The use of soaking in decontamination studies can thus cause misleading results, which cannot be extrapolated to real contamination caused by smoke or indirect contact. The noted differences in contamination show that it is of great importance that, when testing decontamination protocols, contamination procedures are well evaluated in terms of realism and contamination levels, and the use of drug user hair samples is a prerequisite.

New approaches in hair analysis methods

In **Chapter 3**, a completely novel approach to analyze hair samples is proposed: the direct analysis of intact locks of hair using desorption and ionization of analytes by a gas beam consisting of metastable helium species. Where conventional LC−MS/MS and GC−MS/MS involve extensive sample preparation including complete digestion of the hair and/or extraction of the compounds of interest, no sample preparation, apart from decontamination to reduce the risk of false-positive results by external contamination, is needed for DART hair scanning. Hair analysis using DART not only tremendously reduces the analysis time, it also yields continuous timelines of drug exposure without segmentation of the hair sample.

When compared to other recently developed hair analysis methods using MS imaging, the DART hair scan method has two main advantages. As noted before, neither sample preparation nor chromatographic separation is necessary, while hair samples need to be covered with matrix prior to MALDI and MetA-SIMS analysis. Inhomogeneous matrix application can be an additional source of error, matrix signals can interfere with low-mass analytes and the extraction efficiency of drugs of abuse from hair is probably poor.[1] More importantly, analysis of a complete lock of hair, consisting of 150−200 hairs, circumvents the problems caused by asynchronous hair growth cycles observed in single hair analysis. The spatial resolution of MALDI, MetA-SIMS and LAESI methods is far superior to the spot size of DART, making those methods very suitable for studying individual hairs, while analysis of a whole lock of hair is much faster using the DART hair scan method. In this respect, DART hair scanning and high spatial resolution MS imaging methods are complementary. It can be envisioned that, on the one hand, high spatial resolution MS imaging can be used to further investigate micrometer scale processes, such as incorporation pathways and decontamination procedures. On the other hand, DART hair scanning could be applied as a fast hair analysis method for routine use in forensic practice. The lower cost of a DART source compared to a MALDI or MetA-SIMS set-up is an additional benefit for wide-spread forensic applications of the DART hair scan method.

The initial DART hair scan method as proposed in Chapter 3, however, should be regarded as proof-of-principle: only a single drug of abuse was monitored, THC, and the sensitivity was not in compliance with the SoHT-recommended cut-off value. **Chapter 4** describes a greatly improved method involving simultaneous untargeted high-resolution full-scan analysis and data-dependent product ion scans for confirmation of identity of four commonly used drugs of abuse: cocaine, THC, amphetamine, and MDMA. The sensitivity of the DART hair scan method was found to be sufficient for the analysis of cocaine, even below the SoHT cut-off value. In addition, the DART spot size could be reduced using a high spatial resolution exit cone, corresponding to 10 days of hair growth. Where DART cannot compete with MS imaging methods in terms of spatial resolution, the untargeted approach meets the need for retrospective detection of new compounds. The recent trend of use of new psychoactive substances and designer drugs demands continuous evolution of the analytical methodology. The list of compounds used for retrospective interrogation of the high-resolution data can be continuously expanded, and can therefore be used to reevaluate the analysis results for additional positive hits even years after the actual analysis.

The manner of how targeted drugs of abuse are used leads to different demands on hair analysis methods. Cannabis, for example, is mostly continuously, that is daily or several times a week, used, while so-called party drugs like MDMA and ketamine will generally be used less often and thus yield different exposure timelines. An even more specific temporal profile might be obtained from hair samples after involuntary administration of a date rape drug such as GHB. To determine what retrospective timeline precision is desired for optimal use of a hair analysis method in forensic practice, a closer look into the incorporation of compounds into the hair matrix is needed. As described in Chapter 1, many factors and mechanisms are involved in the accumulation of compounds in hair. Recent literature reported variations within longitudinal temporal drug profiles depending on the properties of the detected compound and its incorporation mechanism, even when drug intake is very similar. Constant positive segments along the hair were found in a sample taken 30 days after a single dose of tilidine. This was most likely caused by incorporation of tilidine from sweat into the hair matrix.[8] In contrast, characteristic clozapine administration cycles could be obtained from hair samples of a poison victim, resulting in a very precise administration timeline.[9] Recent MALDI imaging of longitudinal sections of scalp hair shafts showed two incorporation sites for the incorporation of methoxyphenamine into the hair matrix: the hair bulbs and the upper dermis area. Each incorporation site resulted in a 2−3 mm band, which grew close together along the hair shaft toward its distal end. Consequently, a single dose drug intake of methoxyphenamine resulted in an experimental chronological resolution of about 11 days.[10] From the discussed findings, it can be stated that, physiologically, retrospective assessment of time of drug administration will at best have an accuracy of 1−2 weeks instead of days. Conventional hair analysis methods using 1−3 cm segments, resulting in timeline zones of 1−3 months, thus providing inadequate accuracy. A spatial resolution corresponding to hours or even minutes[11] of hair growth, obtained by MS imaging of single hairs does not result in a timeline with corresponding accuracy because the limiting factor will be the incorporation rates mentioned. In between those two extremes, DART hair scanning will add some uncertainty to the timeline accuracy by its spot size corresponding to 10 days of hair growth when a high resolution exit cone is used. However, the total retrospective timing error using the DART hair scan method is still in the order of weeks. Combined with the short analysis time and analysis of a complete lock of hair, the DART hair scan method should be considered as a promising intermediate between conventional hair analysis and cutting-edge high spatial resolution MS imaging.

As discussed in Chapter 1, ambient ionization techniques come with different drawbacks such as a limited *m/z* range, high standard deviations or difficult use of internal standards. The *m/z* range of DART, roughly from *m/z* 50 up to 1200, was found to be sufficient for the analysis of drugs of abuse since the great majority of the compounds used for retrospective interrogation of the full-scan accurate mass data in Chapter 4 had a molecular weight between 150 and 400 Da. While an internal standard could be used to correct for spiking inconsistencies when using the TLC sprayer (Chapter 3), no compounds can be added to the inside of a hair. Therefore, it is not possible to use internal standards to reduce the standard deviation for quantification in DART hair scanning. Additionally, the absent sample preparation, one of the advantages of DART hair scanning, results in ionization of many compounds in and on hairs and relatively high standard deviations. Because of the lack of internal standards and the high standard deviations, no absolute quantification was achieved using the DART hair scan method, as noted in Chapter 4. However, an estimation of the drug concentration in a hair sample can be obtained. For cocaine, for example, a 150 times higher concentration (74.8 vs. 0.5 ng/mg) in authentic drug user hair samples yielded an approximately 200 times higher DART-HRMS signal: 7.2×10^7 vs. 3.6×10^5 area counts/mm of hair. Even though no absolute numbers can be obtained, these results, together with the relative calibration curves presented in Chapter 3 and the analysis of spiked and drug user hair samples presented in Chapters 3 and 4, show that the DART hair scan method is able to yield approximate drug levels. In a broader context, these findings are in good correspondence with literature reporting on quantification using DART, confirming its biggest drawback: without the use of an internal standard, RSDs are too high for quantification.[12-14]

The comparison of different MS instruments regarding their selectivity and sensitivity for the direct analysis of hair samples described in **Chapter 5**, underlines the necessity of careful evaluation of hair analysis results obtained by direct or ambient MS techniques. More research is needed to completely understand the MS/MS results obtained by the triple quadrupole instrument. Nevertheless, the conclusions of this study can probably be extrapolated to many other direct and ambient MS techniques ionizing a complete sample with limited sample cleanup.

With DART-HRMS(/MS), no differentiation could be made between THC and CBD, because both compounds have the same elemental composition and yield the same product ions. Since ambient MS techniques generally lack a separation step such as chromatography, isomers with similar fragmentation behavior cannot be distinguished. The use of TWIM to separate compounds based on their molecular size and shape, as described in Chapter 5 for the separation of THC and different isobaric ions, was also investigated to separate THC and CBD. Unfortunately, these two cannabinoids are very much alike in terms of molecular geometry and no significant separation could be achieved using TWIM. Ion mobility separation can be enhanced by formation of metal adducts,[15] however, DART ionization does not yield metal adducts. Another approach to differentiate between THC and CBD is via H-D exchange. Because THC has only one exchangeable hydrogen atom at the hydroxyl group and CBD has two, H-D exchange would result in a mass difference of 1 Da. To induce H-D exchange in DART ionization, deuterium has to be introduced into the ionization region as previously reported.[16] The required mass resolution to separate deuterated CBD from the ^{13}C isotope of THC is above 200,000 FWHM. Only few MS instruments have such a high mass resolution, thus limiting available instrument choices. This approach has to be tested, but could be a way to differentiate between THC and CBD in DART hair scanning.

Chapter 4 describes two procedures for DART hair analysis: longitudinal scanning of intact locks of hair and ultrafast untargeted screening of hair samples by holding a hair sample in the DART ionization region for a few seconds. The main difference between both procedures is the possibility of retrospective assessment of time of drug exposure, which is absent when the ultrafast approach is used. The ultrafast screening approach is not intended for analysis of a complete hair sample since only a small part is exposed to the DART gas stream. This requires some care in selection of the hair sample, but the ultrafast screening procedure, for example, enables rapid analysis of the most proximal part of the hair to detect recent drug use. Direct analysis using tweezers will reduce analysis time compared to longitudinal scanning of a sample because the sample does not have to be attached to a holder. The ultrafast screening approach is therefore especially convenient in cases where no timeline information is needed or possible, for example when only short hairs can be sampled or a hair sample is not aligned due to improper sampling or storage.

Application of forensic hair analysis protocols to veterinary drug residue analysis

Next to evaluation of decontamination procedures and development of a new hair analysis technique, the use of forensic hair sampling and segmentation for retrospective assessment of time of treatment in veterinary control was investigated. The results described in **Chapter 6** show that by using the sampling and segmentation protocols recommended by the SoHT,[17] an estimated time of drug administration can be obtained with an error in the order of weeks. With the current demand for a safe and animal- friendly food industry, timeline information regarding drug treatment of cattle is required for frequently traded livestock to be able to identify the actual party responsible for use of controlled or banned substances. Although hair analysis without segmentation has already been used for controlling substance abuse, the findings described in Chapter 6 are an important step forward towards accurate determination of time of administration. Not only can hair analysis methods using segmentation be used to detect peak levels in drug profiles throughout the hairs, indicating drug administration for a short time period as in Chapter 6, it also uses smaller sample amounts enabling use of the method even when the sample amount is small. Chapter 6 demonstrates the feasibility of segmented hair analysis for control of the use of clenbuterol, but the same methodology can be extended to many more controlled or banned substances when assessment of time of drug administration is required. Furthermore, the same decontamination issues as in human forensics apply to veterinary hair samples since contamination of hair is possible via sweat or licking upon drinking substance-containing milk.[18]

As noted in the conclusion of Chapter 6, the DART hair scan method should also be applicable to veterinary hair samples. Especially ultrafast untargeted DART screening, as described in Chapter 4, could be of great value for routine hair analysis of controlled or banned substances when no timeline information is required. The feasibility of DART to detect clenbuterol in bovine hair was investigated by analysis of standard solutions and authentic hair samples. DART analysis of clenbuterol standard showed an isotope pattern as expected from the presence of two chlorine atoms (Figure 3A). Three hair samples containing approximately 0.1 ng clenbuterol/mg were analyzed by use of DART hair scanning. One sample resulted in the detection of clenbuterol in bovine hair, as shown in Figure 3B. Even though clenbuterol was detected in this sample by DART scanning, previous LC−MS/MS analysis showed that clenbuterol levels are generally lower and only up to 0.03 ng/mg (Chapter 6). The concentration of incorporated clenbuterol in hair is thus low compared to many human drugs of abuse and to be able to apply DART hair scanning to routine hair testing for clenbuterol, a more sensitive set-up will be needed. Nevertheless, the use of the DART hair scan method for veterinary samples will achieve the same advantages as in forensic practice: less sample preparation, shorter analysis times, and, when scanned, continuous timeline information. When full-scan high-resolution data is obtained, retrospective data analysis can be used leading to fast, untargeted analysis of veterinary hair samples.

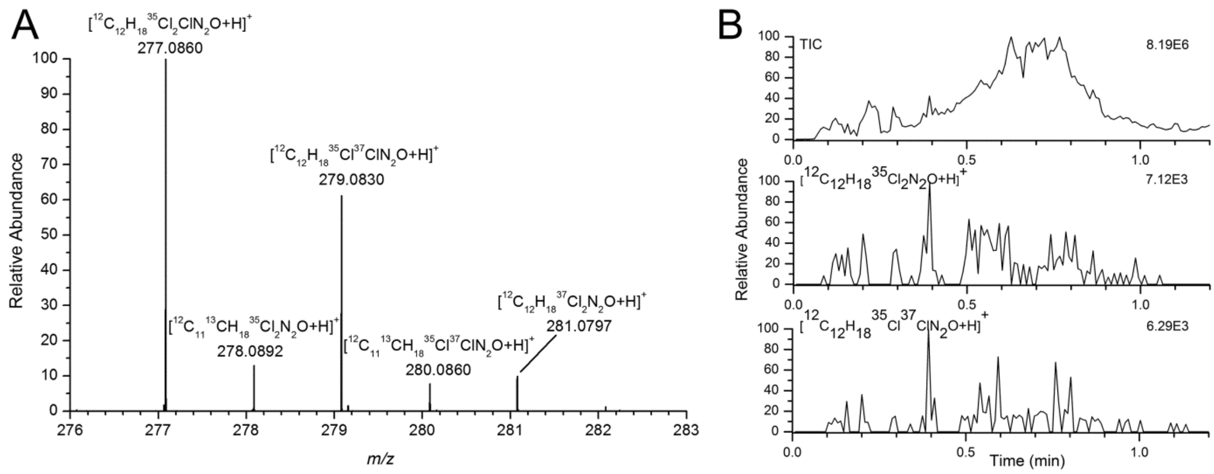

Figure 3. *A) Positive DART-quadrupole orbitrap MS scan of clenbuterol standard solution. B) DART orbitrap MS total ion current (TIC) and extracted ion chronograms (EIC) of m/z 277.0860 and m/z 279.0830 from longitudinal hair scans of a bovine hair sample.*

Future perspectives

The - in this thesis - developed DART hair scan method has been employed for the analysis of drugs of abuse and occasionally abused pharmaceutical drugs. Since many other compounds can also be incorporated into hair, DART hair scanning has great potential for use in other fields of hair analysis, for example: monitoring therapeutic treatment over a longer period of time,[19,20] or assessment of an individual's well-being by the analysis of the stress hormone cortisol to identify chronic stress.[21] Further applications of DART hair scanning can be found in monitoring an individual's environmental exposure to, for example, pesticides, and in the detection of doping use in sports. Since compounds on the outside of the hair are also ionized by DART, use of the DART hair scan method, without decontamination step, to target compounds exclusively originating from the environment could be envisioned to prove exposure to explosives, or to place an individual in the vicinity of an arson crime scene.

Next to hair analysis, DART, and ambient ionization in general, has been used to analyze a wide range of compounds and matrices in a forensic setting. To this day, however, ambient ionization techniques have not been widely accepted for routine use in forensic laboratories, mostly hampered by the previously described drawbacks, such as challenging quantification, and lack of field testing and fully validated protocols.[22] The implementation of ambient ionization techniques could improve by extensive testing of the developed methods with authentic forensic samples in a non-standardized environment. This will require extensive collaboration of forensic institutes with the manufacturers and the academic world. The current development of miniaturized and portable MS instrumentation, for example, can stimulate field applications of ambient ionization techniques.[22] More extensive testing using authentic drug user hair samples is also needed to validate the DART hair scan method for use in forensic institutes.

To be able to validate a hair analysis method, there is a great need for well-defined positive hair samples. Up till now, only cut or powdered drug user hair is commercially available for hair testing, while intact hair is needed to validate MS imaging methods and the DART hair scan method. Controlled administration of drugs of abuse to individuals to obtain positive hair samples is not possible due to ethical reasons, and therefore substitutes for authentic hair reference samples are desired. The contamination and decontamination results discussed above show that spiked, smoke, or indirectly contaminated hair is different from authentic drug user hair samples. The contamination on and inside soaked hair was found to be more radical and persistent even after repeated wash steps, implying deeper incorporation of compounds into the hair matrix thus possibly offering a means of producing reference samples. Although not tested, SFE might also be useful to transfer non-polar compounds deeper into hairs by addition of the compounds to the supercritical carbon dioxide, thereby mimicking drug user hair samples. A similar result might be achieved by adding drugs of abuse to solvents used in ASE. When deep contamination is followed by proper decontamination to remove compounds from the outer regions of the hair, this procedure might result in drugs of abuse only present inside hairs. The success of this procedure can be tested by MetA-SIMS analysis of longitudinal and cross-sectioned hair to confirm the location of the drugs of abuse along the hair and by quantification using conventional LC−MS/MS or GC−MS/MS analysis after full digestion of the samples to release all compounds from the hair matrix. Critical comparison with authentic drug user hair in terms of drug localization will be needed before the obtained hair material could be used as a standardized alternative for drug user hair.

Little is known about the stability of compounds incorporated into hair. Because variations in caffeine and ethyl glucuronide concentration between locks of hair from the same individual of up to 62% have been reported,[23] proper evaluation of drug concentrations in hair over a long period of time is difficult: small changes in concentration will not be noticed. With standardized hair material, it would be easier to study storage stability. Additionally, different conditions, such as temperature, humidity and exposure to light, could be tested to establish an optimal storage condition.

During the research described in this thesis, another novel forensic application of DART was briefly explored: identification of make-up traces on fabrics. This could, for example, be of use when a victim is suffocated using a pillow or clothes, during which some of the make-up of the victim is transferred to the murder weapon. To be able to identify unknown stains on materials such as a pillow or clothes, different make-up products for use around the eyes, e.g., mascara and eyeliner, from different manufacturers were analyzed using DART-HRMS both as such and after application to fabrics. The mass spectra from make-up on a fabric (Figure 4) resulted in a mixed profile of ions originating from the make-up (Figure 4; indicated by an asterisk) and ions originating from the sample matrix: the fabric (Figure 4; indicated by a number sign). The direct ionization of analytes from a piece of fabric without the need of extraction or other sample preparation could enable fast differentiation between different make-up products and other stains. In addition, the non-destructive nature of DART ionization allows the use of other methods after DART analysis for additional examination of the sample. After further investigation and validation using real evidence, this approach could be used when unknown stains are found on fabric during a forensic investigation and demonstrates the potential wide use of DART ionization.

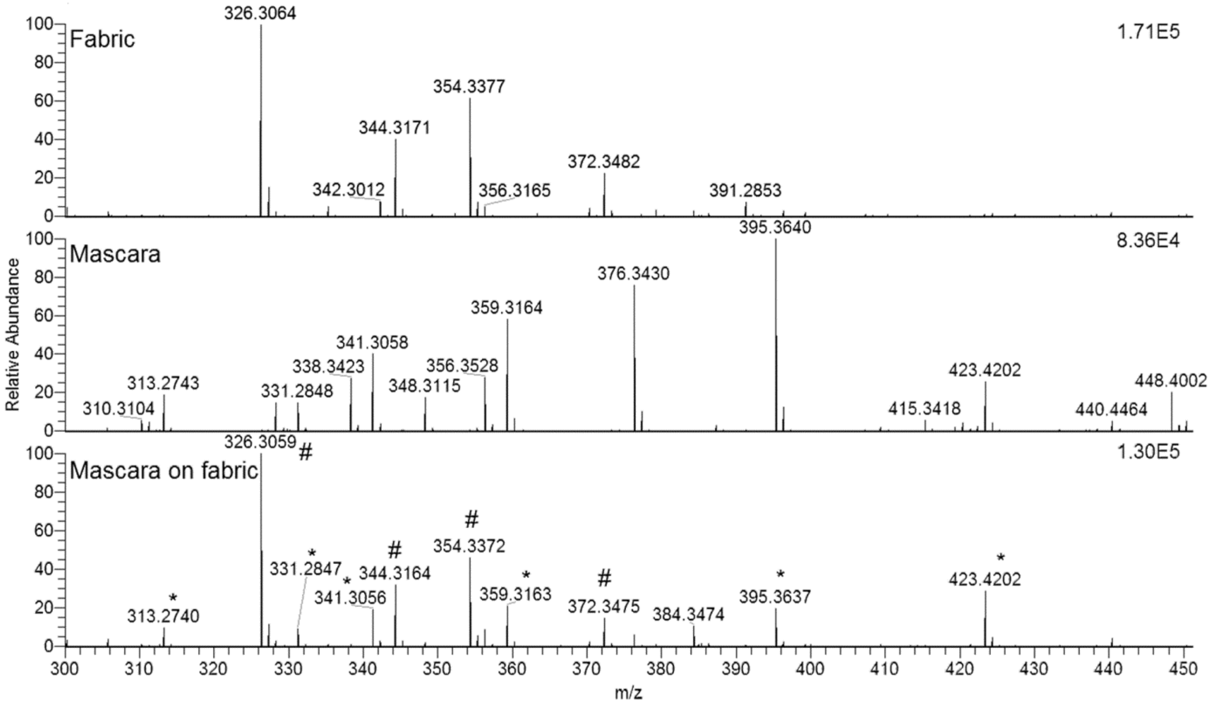

Figure 4. DART-orbitrap MS analysis of a clean fabric (top), mascara (middle), and mascara applied on the same fabric (bottom). Ions originating from the make-up are indicated by an asterisk, ions originating from the fabric are indicated by a number sign.

DART ionization does not only have promising possibilities for the analysis of clenbuterol in hair samples, other applications of DART to the agriforensic field should be possible. A potential use of DART could be the detection of controlled or banned substances from poultry feathers. For this purpose, a similar approach as with hair can be envisioned: scanning of intact feathers to instantly obtain a longitudinal distribution of compounds along the feathers. A recent study by Jansen *et al.*[24] demonstrated the use of segmented analysis of feathers to obtain longitudinal profiles of the antibiotic enrofloxacin. With these profiles, antedating of treatment was possible and differentiation between different types of treatments (therapeutic oral treatment, spray treatment, and sub-therapeutic oral treatment) could be made.[24] The use of DART scanning could shorten analysis times, because less sample preparation will be needed, and more accurate longitudinal profiles would be obtained without the need of segmentation.

Conclusions

Looking back on the main achievements described in this thesis, i.e., evidence-based evaluation of decontamination protocols, development of a novel hair analysis method, and application of forensic protocols to veterinary control, several leads to improve forensic hair evidence are offered. The outcome of this thesis shows that hair analysis is a very viable tool in forensic toxicology and veterinary control, and can be improved by studying and implementing recent analytical innovations. An ideal protocol for forensic practice would involve an evidence-based decontamination step or static DART measurement to differentiate between external and incorporated compounds, followed by longitudinal scanning using DART coupled to a TWIM – quadrupole orbitrap MS instrument with a mass resolution above 60,000 FWHM. When needed, the same hair sample can afterwards be used for confirmatory testing by segmentation and a highly sensitive, quantitative, conventional hair analysis method. In a broader perspective, ambient ionization techniques have an immensely broad application range, of which the research presented in this thesis represents a small part. The range of ambient ionization applications will continue to expand over the years to come.

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Summary

Forensic hair analysis can be used as alternative evidence next to body fluids, and to obtain retrospective timeline information of an individual's drug exposure. **Chapter 1** describes the general concepts of drug incorporation into hair, external contamination, and the current status and limitations of hair analysis methods are introduced. Furthermore, an overview of ambient ionization techniques is given, with emphasis on direct analysis in real time (DART). The instrumentation, ionization mechanisms, and application range of DART are presented. Scientific challenges and objectives to improve forensic hair evidence are formulated, which formed the basis of the research presented in this thesis.

A major issue in forensic hair analysis is the possibility of false-positive results due to external contamination. In **Chapter 2**, an evidence-based evaluation of decontamination protocols for the removal of cannabinoid contamination is presented, mainly focused on Δ^9-tetrahydrocannabinol (THC). Different solvents were extensively tested for their ability to remove cannabinoid contamination originating from cannabis smoke or indirect contact with cannabis plant material. After selection of the most efficient solvents, different sequential wash steps were tested on externally contaminated blank hair samples. Finally, application of the three best performing protocols on cannabis users' hair, both as such and after deliberate contamination, resulted in removal of all contamination without removing incorporated THC. From the detailed scientific evidence reported in this chapter, a protocol using a single methanol wash followed by a single aqueous SDS solution is recommended to remove external cannabis contamination.

A novel approach for the analysis of intact locks of hair consisting of DART combined with high resolution mass spectrometry (HRMS) is developed in **Chapter 3**. DART–HRMS settings were optimized for the analysis of THC and the accuracy of the probed hair zone was investigated using spiked blank hair samples. Intact locks of hair could be longitudinally scanned without the need of extensive sample preparation, resulting in analysis times of only minutes. Detection of THC was achieved in several hair samples from cannabis users. A quantitative liquid chromatography (LC)–MS/MS method was developed, in-house validated, and used to confirm the presence of THC in drug user hair samples. With a retrospective timeline accuracy of ±2 weeks, a significant improvement over conventional segmented hair analysis was achieved. Moreover, differentiation between zones of different THC content within a DART hair scan could be made, indicating possibilities for retrospective assessment of time of drug use.

The DART hair scan method has been improved and expanded in **Chapter 4**. Targeted detection of four commonly used drugs of abuse (amphetamine, cocaine, MDMA and THC) with structural confirmation was achieved by data-dependent product ion scans. Simultaneously, full-scan high-resolution data was obtained and retrospectively interrogated versus a list of more than a hundred, less common, drugs of abuse and occasionally abused pharmaceutical drugs. The hair scan method was validated for the analysis of cocaine against an accredited LC–MS/MS method and the detection limit for cocaine was found to comply with the cut-off value of 0.5 ng/mg. Hair samples of 10 different drug users were analyzed. Next to detection of the four targeted drugs of abuse, retrospective data interrogation revealed several additional hits. The detected substances correlated well with reported drug use and by the detection of several metabolites, drug use could be unambiguously proven. The retrospective timeline accuracy was further improved by use of a high spatial resolution DART exit cone, which yielded a DART spot size corresponding to approximately 10 days of hair growth.

When direct and/or ambient ionization techniques are used to analyze intact hair samples, endogenous isobaric ions can overlap with compounds of interest and yield false-positive results. The selectivity of four MS instruments with different mass analyzers (orbitrap, quadrupole orbitrap, triple quadrupole, time-of-flight) was evaluated in **Chapter 5** by DART analysis of THC from hair samples. To avoid overlap of THC with isobaric ions originating from the hair matrix, a mass resolution of at least 30,000 FWHM was necessary. The use of travelling wave ion mobility spectrometry (TWIMS) resulted in increased selectivity by separation of isobaric ions based on their drift times. A triple quadrupole instrument in multiple reaction monitoring (MRM) mode was found to have the best sensitivity, however, the used transitions were not specific enough for use on drug user hair samples. Thus the selectivity needed to indisputably differentiate THC from endogenous isobaric ions in drug user hair samples could only be achieved by the high resolution of the tested orbitrap MS instruments.

Chapter 6 demonstrates the application of forensic hair analysis techniques to veterinary control. Timeline information could be obtained from veterinary hair samples. For this purpose, a UPLC–MS/MS hair analysis method was adapted and optimized for smaller sample sizes. After validation of the method, segmented hair samples obtained from clenbuterol-treated calves using the forensic hair sampling protocol were analyzed and clenbuterol concentration profiles along the hair samples could be obtained. Assessment of the average growth rate of calf tail hair enabled retrospective determination of time of clenbuterol administration.

The estimated time of administration was reproducible when analyzing sub-samples taken from the same lock of hair and duplicate locks of hair, and in good correlation with the actual treatment.

Through the research presented in this thesis, novel approaches in hair analysis have been developed and the value of forensic hair evidence improved considerably. In **Chapter 7**, the main achievements of this thesis are discussed in detail and an insight in the future perspectives of hair analysis and ambient ionization is given. Potential further applications of the DART hair scan method, and ambient ionization in general, are presented, including some preliminary results of new decontamination strategies, hair analysis possibilities, and other forensic uses of DART ionization.

Samenvatting

Analyse resultaten van haar kunnen worden gebruikt als alternatief forensisch bewijs naast resultaten afkomstig van de meer gebruikelijke analyses van lichaamsvloeistoffen. Daarnaast kunnen haaranalyses informatie verschaffen over iemands drugsverleden. **Hoofdstuk 1** introduceert de algemene concepten van de opname van stoffen in haar, externe contaminatie en de huidige staat en beperkingen van haaranalyse methodes. Daarnaast wordt een overzicht gegeven van zogenoemde ambient ionisatie technieken, met een nadruk op direct analysis in real time (DART). De instrumentatie, ionisatie mechanismen en het toepassingsgebied van DART worden gepresenteerd. De in dit hoofdstuk geformuleerde wetenschappelijke uitdagingen en doelen om forensisch haarbewijs te verbeteren vormen de basis voor het onderzoek dat in de rest van dit proefschrift wordt beschreven.

Een groot probleem in forensische haaranalyse is de kans op vals-positieve resultaten veroorzaakt door verontreiniging van buitenaf, ook wel externe contaminatie genoemd. In **Hoofdstuk 2** worden diverse decontaminatie protocollen voor het verwijderen van cannabis contaminatie in de praktijk vergeleken, met bijzondere aandacht voor Δ^9-tetrahydrocannabinol (THC). Verschillende oplosmiddelen zijn uitvoerig getest op hun vermogen om cannabis contaminatie afkomstig van cannabis rook of indirect contact met cannabis plantmateriaal te verwijderen. Met de meest efficiënte oplosmiddelen zijn verschillende opeenvolgende wasstappen getest op extern gecontamineerde blanco haarmonsters. De toepassing van de drie beste protocollen op haar van cannabisgebruikers, zowel als zodanig als na bewuste contaminatie, resulteerde in verwijdering van alle THC op het haaroppervlak zonder het verwijderen van uit het lichaam opgenomen THC binnenin de haar. Uit de gedetailleerde wetenschappelijke bewijzen in dit hoofdstuk wordt een protocol van een enkele methanol wasbeurt gevolgd door een enkele wasbeurt met waterige SDS-oplossing aanbevolen voor het verwijderen van externe cannabis contaminatie.

Een nieuwe benadering voor de analyse van intacte haarlokken bestaande uit DART ionisatie in combinatie met hoge resolutie massaspectrometrie (HRMS) wordt beschreven in **Hoofdstuk 3**. DART-HRMS instellingen zijn geoptimaliseerd voor de analyse van THC en de nauwkeurigheid van de gemeten haarzone is onderzocht met behulp van blanco haren waarop aan de buitenkant een nauwe zone van cannabis extract is aangebracht. Intacte haarlokken zijn longitudinaal gescand zonder uitgebreide monstervoorbereiding, resulterend in analysetijden van slechts enkele minuten. THC werd gedetecteerd in haarmonsters van diverse cannabisgebruikers. Een kwantitatieve vloeistofchromatografie (LC)–MS/MS methode is ontwikkeld, gevalideerd en gebruikt om de aanwezigheid van THC in haar van drugsgebruikers te bevestigen. Met een retrospectieve tijdlijn nauwkeurigheid van ongeveer 2 weken, is een aanzienlijke verbetering ten opzichte van conventionele gesegmenteerde haaranalyse bereikt. Bovendien konden zones met verschillend THC-gehalte binnen een DART haarscan van elkaar worden onderscheiden, wat mogelijkheden biedt voor retrospectieve bepaling van het moment van drugsgebruik.

De DART haarscan methode is verbeterd en uitgebreid in **Hoofdstuk 4**. Gerichte detectie van vier veelgebruikte drugs (amfetamine, cocaïne, MDMA en THC) met structurele bevestiging is bereikt door middel van data-afhankelijke product ionen scans. Tegelijkertijd zijn hoge resolutie full-scan data verkregen en met terugwerkende kracht onderzocht aan de hand van een lijst van meer dan honderd, minder vaak voorkomende, drugs en misbruikte geneesmiddelen. De haarscan methode is gevalideerd voor de analyse van cocaïne door vergelijking met een geaccrediteerde LC−MS/MS methode en de detectielimiet voor cocaïne voldoet aan de cut-off waarde van 0,5 ng/mg. Haarmonsters van 10 verschillende druggebruikers zijn geanalyseerd. Naast detectie van de vier veelgebruikte drugs, resulteerde retrospectieve data analyse in een aantal extra hits. De gedetecteerde stoffen kwamen goed overeen met het gerapporteerde drugsgebruik en met de detectie van verschillende metabolieten kon drugsgebruik ondubbelzinnig worden bewezen. De retrospectieve tijdlijn nauwkeurigheid is verder verbeterd door het gebruik van een hoge resolutie DART opening, resulterend in een nauwkeurigheid van ongeveer 10 dagen haargroei.

Wanneer directe en/of ambient ionisatietechnieken gebruikt worden om intacte haarmonsters te analyseren, kunnen endogene isobare ionen overlappen met de stoffen waarop de analyse gericht is. Dit kan leiden tot vals-positieve resultaten. De selectiviteit van vier MS instrumenten met verschillende massa analysatoren (orbitrap, quadrupool orbitrap, triple quadrupool, time-of-flight) is geëvalueerd in **Hoofdstuk 5** door DART analyse van THC in haarmonsters. Om overlap van THC met isobare ionen afkomstig uit de haarmatrix te voorkomen, is een massa resolutie van minimaal 30.000 FWHM noodzakelijk. Het gebruik van zogenoemde travelling wave ion mobility spectrometry (TWIMS) resulteerde in een verhoogde selectiviteit door het onderscheiden van isobare ionen op basis van hun drifttijden. Een triple quadrupool instrument in multiple reaction monitoring (MRM)-modus had de beste gevoeligheid, maar de gebruikte ionenovergangen waren niet specifiek genoeg voor gebruik op haarmonsters van drugsgebruikers. De selectiviteit die nodig is om THC onmiskenbaar te onderscheiden van endogene isobare ionen in drugsgebruikers haarmonsters kon alleen worden bereikt door de hoge resolutie van de geteste orbitrap massa spectrometers.

Hoofdstuk 6 toont de toepassing van forensische haaranalyse technieken in veterinaire controle, waarmee tijdlijn informatie is verkregen van veterinaire haarmonsters. Hiervoor is een UPLC−MS/MS haaranalysemethode aangepast en geoptimaliseerd voor kleinere monsterhoeveelheden. Na validatie van de methode zijn haarmonsters, verkregen van clenbuterol behandelde kalveren met het forensische haar bemonsteringsprotocol, gesegmenteerd en geanalyseerd. Clenbuterol concentratieprofielen over de lengte van de haarmonsters zijn, samen met de gemiddelde groei van kalf staarthaar, gebruikt voor retrospectieve bepaling van het tijdstip van clenbuterol toediening. Het geschatte tijdstip van toediening was reproduceerbaar bij het analyseren van duplo haarlokken en sub-monsters genomen uit dezelfde haarlok, en was in goede overeenstemming met de daadwerkelijke behandeling.

Met het in dit proefschrift beschreven onderzoek zijn nieuwe benaderingen voor haaranalyse ontwikkeld. Door toepassing van deze nieuwe technieken kan de waarde van forensisch haarbewijs aanzienlijk verbeterd worden. In **Hoofdstuk 7** worden de belangrijkste resultaten van dit proefschrift in detail besproken en worden de toekomstperspectieven van haaranalyse en ambient ionisatie geschetst. Mogelijke verdere toepassingen van de DART haarscan methode en ambient ionisatie in het algemeen zijn beschreven, waaronder een aantal voorlopige resultaten van nieuwe decontaminatie strategieën, verdere mogelijkheden van haaranalyse en andere forensische toepassingen van DART ionisatie.

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Wilco

Curriculum Vitae

Wilco Franciscus Duvivier was born on the 13th of July 1987 in Koudekerke, The Netherlands. After graduating from high school (SSG Nehalennia, Middelburg) in 2005, he started the bachelor Applied Chemistry at the Hogeschool Zeeland (Vlissingen). As part of this program, he followed the pre-master program 'Chemistry and Physics' at Utrecht University and did an internship at DSM Food Specialties in Delft. During this internship, he developed an interest in mass spectrometry and after obtaining his bachelor's degree, he decided to continue in the field of analytical chemistry by starting the MSc program Analytical Sciences at the University of Amsterdam. He spent part of his MSc program as an exchange student at Københavns Universitet (Denmark) and did his research project at Leiden University in the Analytical BioSciences group. While finishing his MSc, he returned to the R&D department of DSM Food Specialties in Delft to work as a Senior Technician Analysis. In April 2012 he left DSM to start his PhD at the Laboratory of Organic Chemistry at Wageningen University under the supervision of Prof. Dr. Michel Nielen and Dr. Teris van Beek, the results of this work are described in this thesis. Wilco currently works as researcher in the business unit Veterinary Drugs at RIKILT, Wageningen UR.

List of publications

W.F. Duvivier, T.A. van Beek, E.J.M. Pennings, M.W.F. Nielen, Rapid analysis of Δ-9-tetrahydrocannabinol in hair using direct analysis in real time ambient ionization orbitrap mass spectrometry, Rapid Communications in Mass Spectrometry, 28 (7), **2014**, 682- 690.

W.F. Duvivier, T.A. van Beek, T. Meijer, R.J.P. Peeters, M.J. Groot, S.S. Sterk, M.W.F. Nielen, Ultratrace LC–MS/MS analysis of segmented calf hair for retrospective assessment of time of clenbuterol administration in agriforensics, Journal of Agricultural and Food Chemistry, 63 (2), **2015**, 493-499.

W.F. Duvivier, R.J.P. Peeters, T.A. van Beek, M.W.F. Nielen, Evidence based decontamination protocols for the removal of external Δ9-tetrahydrocannabinol (THC) from contaminated hair, Forensic Science International, 259, **2016**, 110-118.

W.F. Duvivier, M.R. van Putten, T.A. van Beek, M.W.F. Nielen, (Un)targeted Scanning of Locks of Hair for Drugs of Abuse by Direct Analysis in Real Time-High-Resolution Mass Spectrometry, Analytical Chemistry, 88 (4), **2016**, 2489-2496.

W.F. Duvivier, T.A. van Beek, M.W.F. Nielen, Critical comparison of mass analyzers for forensic hair analysis by ambient ionization mass spectrometry, Submitted to Rapid Communications in Mass Spectrometry.

Overview of Completed Training Activities

Discipline specific activities

Advanced Organic Chemistry (VLAG), Wageningen, The Netherlands, 2012-2014

Symposia NWO Study Group Analytical Chemistry (NWO), Lunteren, The Netherlands, 2012-2013[a]

51st Annual Meeting of TIAFT (TIAFT), Madeira, Portugal, 2013[a]

The Analytical Challenge (KNCV), Utrecht, The Netherlands, 2013[b]

LTG meeting (LTG), London, United Kingdom, 2013[b]

DGMS meeting (DGMS), Frankfurt, Germany, 2014[b]

NVMS 50th Anniversary Congress (NVMS), Rolduc, The Netherlands, 2014[a]

MSc+ ANAC course: Ambient Mass Spectrometry (COAST), Wageningen, The Netherlands, 2014[b]

Forensic PhD Symposium (CLHC), Amsterdam, The Netherlands, 2014[a]

CHAINS (NWO), Veldhoven, The Netherlands, 2014-2015[a]

Ambient Ionisation SIG Meeting (BMSS), London, United Kingdom, 2014[b]

NVMS Spring Meeting (NVMS), Leiden, The Netherlands, 2015[b]

63rd ASMS Conference (ASMS), St. Louis, MO, USA, 2015[b]

The Analytical Challenge (KNCV), Utrecht, The Netherlands, 2015

3rd NVMS-BSMS Conference on Mass Spectrometry (NVMS-BSMS), Rolduc, The Netherlands, 2016[a,b]

General courses

Techniques for Writing and Presenting a Scientific Paper (VLAG), 2013

Project and Time Management (VLAG), 2013

Mini-symposium "How to Write a World-Class Paper" (VLAG), 2013

Communication with the Media and the General Public (VLAG), 2014

Guide to Scientific Artwork (WUR Library), 2015

Career Perspectives (VLAG), 2015

Optionals

Preparation of PhD research proposal, 2012

Colloquia ORC, 2012-2016

Project meetings, 2012-2016

PhD trip ORC to Germany and Switzerland, 2013

Organizing Committee PhD trip ORC to Germany and Switzerland, 2013

PhD trip ORC to Canada, 2015

[a] Poster; [b] Oral presentation

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