Determination of Psychoactive Substances in Hair for Forensic Purposes

Inaugural dissertation

to

be awarded the degree of Dr. sc. med.
presented at
the Faculty of Medicine
of the University of Basel

by

Ulf Christian Juhl	Meier
from Zürich	Zürich

Basel, 2019

Approved by the Faculty of Medicine

On application of

Prof. Dr. med. Dipl. phys. Eva Scheurer

Dr. phil. Franz Dussy

Prof. Dr. rer. nat. Dipl.-Chem. Volker Auwärter

Basel, ……..

……..

Dean

Prof. Dr. Primo Schär

Table of Contents

List of Common Abbreviations

Abbreviation	Meaning
BetmVV	Betäubungsmittelverzeichnisverordnung
BZD-Z	Benzodiazepines/z-Substances
CBD	Cannabidiol
CBN	Cannabinol
DoA	Drugs of Abuse
EDI	Das Eidgenössische Departement des Innern
EtG	Ethyl glucuronide
EWDTS	European Workplace Drug Testing Society
GTFCh	Gesellschaft für Toxikologische und Forensische Chemie
GC	Gas Chromatography
7-Hy-Mitra	7-Hydroxymitragynine
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
SGRM	Schweizerische Gesellschaft für Rechtsmedizin
SoHT	Society of Hair Testing
SPE	Solid phase extraction
THC	Tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol
THC-OH	11-Hydroxy-Δ^9-tetrahydrocannabinol
VRV	Verkehrsregelnverordnung

Acknowledgements

Firstly, I would like to express my thanks and gratitude to my primary advisor Prof. Eva Scheurer for giving me the opportunity to do this doctorate. She gave me the possibility to explore my research interests as independently as possible, while providing valuable advice when needed, allowing me to grow both professionally and personally.

I would also like to thank Dr. Thomas Briellmann for providing valuable guidance in the beginning of my PhD. While many PhD candidates describe the first months of their studies as disoriented stumbling in the dark, the structure he provided helped me immensely in quickly landing on my feet in a new topic.

My sincere thanks go to Dr. Franz Dussy. He was my secondary advisor for the last two years of my PhD studies, but provided valuable guidance during the entire time. Franz was my go to person when I encountered problems in the lab, needed feedback on interpreting data, writing papers, creating posters or presentations, or simply for some good conversation. Franz had an enormous influence on the success of my work and my enjoyment of my time at the Institute of Forensic Medicine and I could not imagine a better advisor and mentor for my PhD studies.

I would like to thank Dr. Katja Mercer-Chalmers-Bender for helping me with my publications and developing my skill as a toxicologist. A big thank you goes to the entire staff of the institute and especially to the forensic toxicology and chemistry team for making my stay such an enjoyable experience.

Last but not least I would like to thank all of my friends and family outside of work; my girlfriend Sabine for always lending an ear when I needed to vent my frustration over failed experiments or when expressing my joy over the more successful ones, my parents for always supporting me and showing great interest in my work; and my brother Niklaus and my friend Pipo, for the great times we shared and providing a valuable outside perspective on things.

Summary

Hair analysis for abstinence testing has been gaining ever more traction as an alternative to classic matrices such as blood or urine due to the many benefits it offers, the most important being a long window of detection. However, hair analysis still suffers from a lack of knowledge concerning the factors that can influence the concentrations ultimately found in the hair. One such influencing factor, and the topic of this thesis, is the sampling location on the head. This topic had been examined by Dussy et al. 2014 in a small study. The study had a small sample size of three test subjects and did not investigate the entire head systematically. Additionally, only ethyl glucuronide (EtG) and caffeine were investigated. Therefore, little could be said about the patterns of distribution or the possible extent of differences, especially about any other substance than EtG and caffeine.

In a first step, the entire scalp hair of a single person was investigated. For this, the scalp hair was divided into individual strands of roughly 3x3 cm area yielding a total of 104 strands across the head. Each strand was analyzed for EtG and caffeine. For this, a method for the analysis of EtG using LC-MS^3 was developed, as the previous employed GC-MS/MS method was not suitable for large series of samples. The complete analysis yielded EtG concentrations between 6.8 and 20.2 pg/mg. For caffeine, values between 1.1 and 12.0 ng/mg were obtained. The distribution patterns of EtG and caffeine were markedly different. While caffeine clearly showed higher concentrations towards the edges of the haircut, EtG was not as clearly distributed except for lower concentration at the neck.

As these results were very promising, the distribution was investigated more thoroughly. Cocaine is the second most important substance for hair analysis after EtG at our lab, as it is the most prevalent in the routine population. Therefore, the study focused on alcohol and cocaine consuming individuals. For each individual, all hair was sampled in the same way as in the previous single person study. As the sample preparation for EtG and drugs of abuse (DoA) was normally done with different methods at our lab, the analysis of the many hundreds to thousands of hair samples would have exceeded the manageable workload. Therefore, a combined sample preparation method for EtG, caffeine, DoA and benzodiazepines/z-substances (BZD-Z) was developed. The method is based on the different retention characteristics of the substances to an Oasis Max SPE cartridge. Using this combined sample preparation, a large workload reduction was possible. Also in cases with little available hair, a full analysis was still possible.

In addition to determining the shape and extent of the substance distributions, finding explanations for the observed differences was of interest. Therefore, in cooperation with the clinic for angiology and the department for sport, exercise and health, the head skin perfusion and sweating rates across the scalp were investigated, as the bloodstream and the sweat are thought to be important incorporation pathways. The head skin perfusion was measured using a laser Doppler anemometer. Each sample area was scanned allowing the same cartography of the perfusion rates as with the substance concentrations. The head skin sweating rates were measured using a cycling cap which was lined with pads of strongly water absorbing material.

The test subjects were asked to cycle for 10 minutes on an ergometer after which the cycling cap was placed on their head and another 10 minutes of cycling were commenced. The weight of the pads was measured before and after cycling to obtain the weight of the excreted sweat.

Thirteen alcohol and/or drug consuming persons, 12 of which were cocaine consumers and 9 of which were alcohol consumers could be enlisted. The distribution patterns for 29 different substances were obtained. For many substances (almost all DoA and BZD-Z) the distribution showed a clear pattern of higher concentrations on the periphery of the head and especially on the forehead. EtG in general showed a reverse behavior to this, with higher concentrations towards the vertex posterior region. The extent of the found differences across the head was depended strongly on substance and on the individual. Cocaine showed factors between 2.8 and 105 (median 8.4) between lowest and highest concentrations, while e.g. methadone showed factors of only 1.6 to 4.2 (median 2.0). EtG showed factors between 2.5 and 7.1 (median 4.0). These differences are very relevant as typically cut-off thresholds are applied when evaluating hair results. This leads to situations in which results of samples from different parts of the head lead to a different evaluation of the consumption habits. In the case of EtG, two thresholds (7 pg/mg [5 pg/mg according to the new SoHT consensus of 06.08.2019] and 30 pg/mg for abstinence and chronic excessive consumption, respectively) are applied. One participant showed concentrations between 6.2 pg/mg and 30.4 pg/mg. For this participant, depending on sampling location, the whole range of interpretations is possible if applying the former cut-offs. For seven of nine study participants, the EtG concentrations fell into more than one category. This is problematic and should be considered when sampling and documenting sampling in routine work. The perfusion measurements did not show a clear pattern of perfusion rates, with only the back of the neck showing significantly lower perfusion rates across participants. The sweat rates showed large differences with sweating being highest on the forehead and generally decreasing towards the vertex posterior. The sweat rates showed a similar behavior to the concentrations of most substances, meaning the concentration differences could be caused by sweating. EtG showed a reverse distribution to the sweat rates, so might instead be washed out by the sweat.

From these results, sweat rate differences across the scalp are believed to be a major source of the observed concentration differences. For sweat to be a major incorporation pathway, substances must on the one hand be able to cross from the bloodstream into the sweat, and on the other be able to go from the sweat into the hair. Most research studying the incorporation of substances from the sweat into the hair were conducted within the context of external contamination. Therefore, these studies usually focused on cocaine and employed high concentrations and unrealistic conditions for modeling the daily incorporation from sweat of a drug user. On this basis, the incorporation of the routine panel of substances into the hair from artificial sweat solution was investigated to test the plausibility of the hypothesis that sweat is a major contributor to the concentration differences found across the head. This project is currently ongoing and definite results are not yet available.

One study participant reported consuming Kratom every day. As mitragynine and 7-hydroxymitragynine (7-Hy-Mitra) have recently been listed as scheduled substances in Switzerland, a method was developed for the measurement of mitragynine and 7-Hy-Mitra in hair and applied to the hair of the study participant. 7-Hy-Mitra could not be detected in any

sample. Mitragynine showed a narrow concentration distribution with concentrations between 1.1 ng/mg and 2.2 ng/mg with no clear pattern. The method was applied to routine drug of abuse hair samples to estimate the prevalence of Kratom consumption. After measurement of 300 routine samples, not a single sample was positive, showing a low prevalence of Kratom consumption.

Recently, hemp material containing less than 1 % THC and high amounts of cannabidiol (CBD) have become available for legal purchase in Switzerland. No information was available if smoking this CBD rich hemp could yield THC concentrations above the legal cut-off of 1.5 ng/mL in blood (2.2 ng/mL with the confidence interval; VRV Art. 2 § 2). Therefore, a self-experiment was conducted to determine if concentrations above this legal limit could be reached. In this experiment, an employee of our institute smoked a CBD joint while blood was collected at regular intervals. Urine was collected from every spontaneous void. To see if an accumulation of THC could occur, the same person smoked two CBD joints per day for ten day. While smoking the last joint, blood and urine were again collected. The experiment showed that blood concentrations above the legal threshold of THC could be achieved with THC concentration of 2.7 ng/mg and 4.5 ng/mg after the single and chronic smoking experiments, respectively. No accumulation of THC in blood or urine could be detected during the 10-day smoking period. A slight accumulation of THC-COOH in urine is possible, but could not be definitely confirmed.

Zusammenfassung

Die Haaranalytik hat sich in der forensischen Toxikologie zunehmend etabliert. Vor allem im Bereich der Abstinenzkontrollen verdrängt die Haaranalytik die klassischen Matrizes Blut und Urin, aufgrund der vielen Vorteile, welche Haare im Vergleich bieten, allen voran das längere Nachweisfenster. Dennoch fehlt einiges an grundlegendem Wissen, was die Einflussfaktoren auf die Substanzkonzentrationen in den Haaren betrifft. Einer dieser Einflussfaktoren ist die Probennahmestelle am Kopf. Die Beschreibung dieses Faktors stellt das Thema dieser Dissertation dar. Dieses Thema wurde von Dussy et. al 2014 in einer kleinen Studie aufgegriffen. Die Studie hatte eine begrenzte Teilnehmerzahl von drei Personen und es wurden lediglich 10 Haarproben pro Kopf asserviert. Auch wurden nur Ethylglucuronid (EtG) und Koffein in der Studie untersucht und folglich konnten keine Aussagen bezüglich der Verteilung von z.B. Betäubungsmitteln gemacht werden.

Um den Verteilungsmustern von Substanzen auf den Grund zu gehen, wurden in einem ersten Schritt die gesamten Kopfhaare eines einzelnen drogenabstinenten Probanden asserviert. Die Asservierung verlief in einem Rasterschema mit Gebieten von jeweils ca. 3x3 cm. Daraus resultierten insgesamt 104 Haarsträhnen, welche alle auf EtG und Koffein untersucht wurden. Die Untersuchung wurde mit einer selbst entwickelten Methode mittels LC-MS^3, welche die zuvor eingesetzte GC-MS Methode ersetzte, durchgeführt. Die gefundenen EtG Konzentrationen lagen zwischen 6.8 pg/mg und 20.2 pg/mg, während die Koffeinkonzentrationen zwischen 1.1 ng/mg und 12.0 ng/mg lagen. Die Verteilungsmuster von EtG und Koffein unterschieden sich grundlegend. Koffein wies klar höhere Konzentrationen an der Peripherie der Frisur auf. Die höchsten Konzentrationen wurden an der Stirn festgestellt. EtG wies hingegen kein klares Muster auf, ausser dass im Nacken deutlich tiefere Konzentrationen festgestellt wurden.

Diese Resultate waren äusserst vielversprechend, weshalb daraufhin eine umfangreichere Untersuchung der Verteilungsmuster ins Auge gefasst wurde. Da in unserem Labor Kokain neben EtG am häufigsten in den Haaren festgestellt wird, wurden Alkohol- und Kokainkonsumenten für eine Studie rekrutiert. Von jedem Teilnehmer wurde die Gesamtheit der Kopfhaare asserviert und auf EtG, gängige Betäubungsmittel (DoA) und Benzodiazepinen/Z-Substanzen (BZD-Z) untersucht. Da von jedem Probanden um die 100 Haarproben erwartet wurden, hätte der zeitliche Rahmen des Projektes eine Analyse auf alle benannten Substanzklassen mit den bestehenden Analysemethoden mit mehrfacher Probenaufarbeitung nicht zugelassen. Deswegen wurde eine kombinierte Probenaufarbeitung für alle drei Substanzklassen entwickelt. Die Methode basiert auf dem unterschiedlichen Retentionsverhalten der DoA und der BZD-Z gegenüber EtG auf einer Oasis Max® SPE Kartusche. Durch den Gebrauch der kombinierten Probeaufarbeitungsmethode konnte der Arbeitsaufwand massiv reduziert werden. Ausserdem war eine vollständige Analyse auch bei geringeren verfügbaren Probenmengen möglich.

Zusätzlich zu der Beschreibung der Verteilungsmuster sollten die Gründe für die beobachteten Verteilungsmuster untersucht werden. Zusammen mit der Klinik für Angiologie

des Unispital Basel sowie dem Department für Sport, Bewegung und Gesundheit wurde eine Kooperation aufgebaut, um die Kopfhautdurchblutungsraten und Schweissraten auf dem Kopf zu untersuchen, da die Einlagerung über die Blutbahn und den Schweiss als wichtige Einlagerungsmechanismen gelten. Die Kopfhautdurchblutung wurde mittels "Laser Doppler Perfusion Imaging" untersucht. Jedes Haarprobennahmegebiet wurde so untersucht, um eine eins-zu-eins Korrelation der Perfusionsdaten mit den Konzentrationsdaten zu erlauben. Die Kopfschweissraten wurden gravimetrisch mit Hilfe einer Fahrradmütze untersucht. Diese wurde mit stark wasseradsorbierenden Pads ausgekleidet. Die Studienteilnehmer mussten 10 Minuten ohne Mütze Fahrrad fahren und anschliessend weitere 10 Minuten mit der Mütze.

Für diese Studie konnten 13 Teilnehmer rekrutiert werden, wovon 12 Kokainkonsumenten und 9 Alkoholkonsumenten waren. Die meisten Studienteilnehmer waren Konsumenten mehrerer DoA bzw. BZD-Z und somit konnten Verteilungsmuster von 29 verschiedenen Substanzen ermittelt werden. Die meisten Substanzen (beinahe alle DoA und BZD-Z) zeigten höhere Konzentrationen am Rande der Frisur. EtG hingegen zeigte oft ein gegensätzliches Verhalten mit höheren Konzentrationen im Vertexbereich. Es gab grosse interindividuelle und substanzspezifische Unterschiede im Ausmass der Verteilung. Zum Beispiel wies Kokain Faktoren zwischen 2.8 und 105 (Mittelwert 17.6) auf, wohingegen Methadon nur Faktoren zwischen 1.6 und 4.2 (Mittelwert 2.4) aufwies. Für EtG wurden Faktoren zwischen 2.5 und 7.1 (Mittelwert 4.4) erhalten. Das Ausmass dieser Unterschiede ist durchaus relevant für die Bewertung von Haarproben. Typischerweise werden Grenzwerte bei der Bewertung von Haarproben angewendet. Unterschiede in dem gefundenen Ausmass können zu Situationen führen, in denen die Haarprobennahme von unterschiedlichen Kopfgebieten zu einer anderen Bewertung führt. Zum Beispiel werden für EtG die beiden Grenzwerte 7 pg/mg (5 pg/mg nach neuem SoHT Konsens 06.08.2019) für den Abstinenznachweis und 30 pg/mg, um zwischen sozialem Trinkverhalten und chronisch exzessivem Trinkverhalten zu unterscheiden, verwendet. In der Tat wies einer der 9 untersuchten Probanden EtG Konzentrationen zwischen 6.2 pg/mg und 30.4 pg/mg auf, womit die gefundenen Konzentrationen bei Verwendung des alten Grenzwertes den gesamten Interpretationsraum abdecken. Sieben der neun Studienteilnehmer wiesen Konzentrationen auf, welche zu mehr als einem Interpretationsbereich gehören. Dies ist natürlich problematisch und sollte während der Probenahme und der Interpretation der Ergebnisse berücksichtigt werden.

Die Perfusionsmessungen zeigten kein klares Muster der Durchblutungsraten über den Kopf auf, obschon die Durchblutung im Nackenbereich tendenziell etwas tiefer war. Im Gegensatz dazu zeigten die Schweisssekretionsraten grosse Unterschiede über den Kopf. Die höchsten Schweissraten wurden an der Stirn gefunden, und generell an der Peripherie der Frisur. Die Schweissraten nahmen in Richtung Vertex ab. Somit zeigten die Schweissraten eine ähnliche Verteilung wie die Konzentrationen von vielen Substanzen, was vermuten lässt, dass die Konzentrationsunterschiede durch die unterschiedlichen Schweissraten verursacht werden könnten. Dabei würden die meisten Substanzen über den Schweiss eingelagert werden, mit Ausnahme von EtG, welches durch den Schweiss eher aus den Haaren rausgewaschen würde.

Falls Unterschiede in den Schweissraten tatsächlich für die Konzentrationsunterschiede mitverantwortlich sind, müssen Substanzen einerseits von der Blutbahn in den Schweiss gelangen können und anschliessend vom Schweiss in das Haar gelangen und dort dauerhaft

eingelagert werden. Die meisten Studien, welche die Einlagerung von Substanzen aus Schweiss in die Haare untersuchen, tun dies im Kontext einer akuten externen Kontamination. Deswegen zielen die Studien meist auf das Kokain und verwenden meist sehr hohe Konzentrationen. Diese Konzentrationen sind unrealistisch in Bezug auf Situationen ohne externe Kontamination und mit dem Hintergrund der Ausscheidung dieser Substanzen durch den Schweiss. Aus diesem Grund wurde eine Studie zur Untersuchung der Einlagerung von Substanzen in die Haare aus einer synthetischen Schweisslösung mit realistischen Konzentrationen entworfen. Die Studie soll die Plausibilität der Hypothese, dass die Verteilungsmuster aufgrund von Unterschieden in den Schweissraten zustande kommen, testen. Diese Studie läuft zurzeit und es können noch keine definitiven Resultate präsentiert werden.

Einer der Studienteilnehmer gab an, täglich Kratom zu konsumieren. Mitragynin und 7-Hydroxymitragynin (7-Hy-Mitra) wurden in der Schweiz kürzlich in das Betäubungsmittelverzeichnis (BetmVV-EDI) aufgenommen. Vor diesem Hintergrund wurde eine Methode zum Nachweis von Mitragynin und 7-Hy-Mitra entwickelt und auf jede asservierte Haarsträhne dieses Konsumenten angewendet. 7-Hy-Mitra konnte in keiner seiner Haarproben nachgewiesen werden. Mitragynin zeigte eine enge Verteilung mit Konzentrationen zwischen 1.1 und 2.2 ng/mg ohne ein erkennbares Muster. Die Methode wurde auf einen Teil der Haare in der Routineanalytik angewendet, um die Prävalenz des Kratomkonsums in unserer Routinepopulation zu untersuchen. Von den 300 bislang gemessenen Haarproben wurde keine der Haarproben positiv auf Mitragynin getestet, was auf eine tiefe Prävalenz für wiederholten/regelmässigen Konsum schliessen lässt.

Kürzlich wurde Cannabis mit einem THC Gehalt von unter 1 % und hohem Cannabidiol (CBD) Gehalt für den legalen Verkauf in der Schweiz freigegeben und ist nun z.B. an Kiosken erhältlich. Es bestand der dringende Bedarf zu überprüfen, ob das Rauchen von CBD Hanf zu Blut-THC-Konzentrationen über dem in der VRV Art. 2 Abs. 2 aufgeführten Grenzwert von 1.5 ng/ml (bzw. 2.2 ng/ml unter Berücksichtigung des Vertrauensintervalls), ab welchem die Fahrfähigkeit aufgrund eines nachgewiesenen THC-Konsums als von Gesetztes wegen als erwiesen gilt, führen könnte. Deswegen wurde ein Selbstexperiment mit dem Ziel durchgeführt, zu überprüfen, ob Blutkonzentrationen über dem Grenzwert erreicht werden könnten. Dazu rauchte eine Mitarbeiterin des IRM Basel einen CBD Joint. Währenddessen wurden in regelmässigen Abständen Blutproben entnommen. Ausserdem wurde für 24 Stunden jeder Spontanurin asserviert. Um zu überprüfen, ob eine Akkumulation von THC bei längerem CBD-Hanf Konsum wahrscheinlich ist, hat dieselbe Mitarbeiterin während 10 Tagen jeden Morgen und Abend jeweils einen Joint geraucht. Beim Rauchen des letzten Joints wurden analog zum ersten Experiment Blut- und Urinproben asserviert. Das Experiment zeigte, dass für eine kurze Zeit nach dem Rauchen Blutkonzentrationen über dem Grenzwert erreicht werden konnten. Die höchsten THC Blutkonzentrationen nach dem ersten und letzten Joint waren 2.7 ng/mg beziehungsweise 4.5 ng/mg. Es konnte keine Akkumulation von THC während der 10-tägigen Rauchphase festgestellt werden. Eine geringe THC-COOH Akkumulation im Urin der Mitarbeiterin könnte stattgefunden haben, die Resultate waren aber nicht hinreichend, um dies mit Sicherheit zu belegen. Solch eine Akkumulation könnte für den Abstinenznachweis mittels Urinproben problematisch sein.

1. Introduction

1.1. An Overview – What Is Hair Analysis, What Is It Used for, and How Is It Done?

Hair analysis has been steadily gaining footing in forensic toxicology as the matrix of choice for abstinence testing and for establishing a history of substance consumption.[1] Hair is different from the classic matrices of choice, urine and blood, in that it has a much longer window of detection. While blood and urine usually cover time windows of a few hours or a few days, respectively, hair covers many weeks to months, allowing for a long retrospective view into the substance uptake history of a person. Hair offers many other advantages over blood and urine. Substances are stable in hair for long term storage if the hair is stored in a dry and dark environment. Hair does not need to be cooled or frozen for storage as urine and blood do, and a neatly packed hair lock takes very little storage space. Additionally, hair sampling is non-invasive compared to urine and blood sampling, and hair samples are much harder to adulterate than urine samples. These advantages make hair analysis very useful and promoted its application for purposes such as workplace drug testing, driver license regranting procedures, doping controls, for determining criminal liability and drug addictions in criminal cases, and child custody cases. While hair analysis has many advantages, it cannot be used to determine acute consumption or exposure to a substance. Also, the results of hair analysis cannot be used to judge the effects a substance had on a person at a specific time point. As useful as hair analysis is, it also suffers from numerous limitations, some of which will be discussed in chapter 1.6. For extensive reviews on hair analysis and its applications refer to Pragst and Balikova 2006, Kintz (Ed.) 2007, and Kintz, Salomone, and Vincenti (Eds.) 2015.[2-4] The first documented case of hair analysis dates back to 1858.[5] The hair of a body that had been exhumed 11 years after death was analyzed and arsenic could be identified. More than a hundred years later in 1979 Baumgartner et al. published a paper describing the determination of the opiate abuse history from radioimmunological analysis of hair.[6] This was the start of modern hair analysis. However, hair analysis really took off with the availability of GC-MS technology, allowing specific identification of substances as well as more sensitive analysis.

Hair analysis typically proceeds via the following steps: First, hair is sampled. This is preferentially done at the vertex posterior of the head. Hair is sampled as close to the skin as possible by cutting a lock of hair using scissors. Afterwards, the hair is cut to a length corresponding to the desired time window (see chapter 1.3). The hair is washed to remove external contamination and after drying is cut into snippets for homogeneity and subsequently sometimes pulverized. In some reported methods, the hairs are dissolved using e.g. sodium hydroxide solutions to increase the extraction yields. The extracts are subjected to varying clean-up procedures such a solid phase extraction (SPE), liquid-liquid extraction, filtration, etc. The extracts are finally measured, typically using GC-MS^n or LC-MS^n. These techniques are firmly established as the Gold Standard for hair analysis in forensic Toxicology.[7]

In the following chapters, hair structure, mechanisms of hair growth, skin anatomy, incorporation of substance into the hair, and pitfalls of hair analysis are briefly reviewed, and the topic of this thesis is introduced.

1.2. Structure of Hair

The structure of the human hair shaft, follicle anatomy, and the mechanism of melanin incorporation into the hair shaft are presented in Figure 1. The hair shaft is built up of three layers; the cuticle, the cortex, and the medulla.[8,9] The *cuticle* is the outermost layer of the hair and consists of layers of cells with a structure resembling reptilian scales or shingles with the scale edges pointing towards the distal end of the hair shaft. Cuticle cells are typically about 0.5 µm thick and have a visible length of about 50 µm. The cuticle has a thickness of about 5 – 10 cuticle cells.[8,10] The cuticle plays an important role in protecting the inner parts of the hair from environmental damage to the hair structure, while being responsible for keeping hair clean, disentangled, and is important for its shine.[2,8] The cuticle is susceptible to damage from a variety of sources such as light, heat, or chemical treatment. With time, the protection provided by the cuticle may be compromised, and deeper hair layers can be exposed to the environment, leading to damaged and frayed hair towards the distal end.[11,12] Below the cuticle is the *cortex* which makes up the large part of the hair. The cortex is responsible for giving the hair its color, structure and flexibility. Cortical cells are long spindle shaped structures that contain hard keratin filaments. A few hundred of these filaments together form larger structures called macrofibrils. Chemically, the hair is to a large part composed of alpha-keratin intermediate filaments and keratin associated proteins, a group of proteins that form a cross-linked network between the filaments.[8,13] Finally, in the center of the hair, is the *medulla* which is often absent or fragmented in human hairs. The medulla is made of a stream of cells that is interspersed with vacuoles. The number of medullar cells typically increases with increasing hair diameter.[9,10]

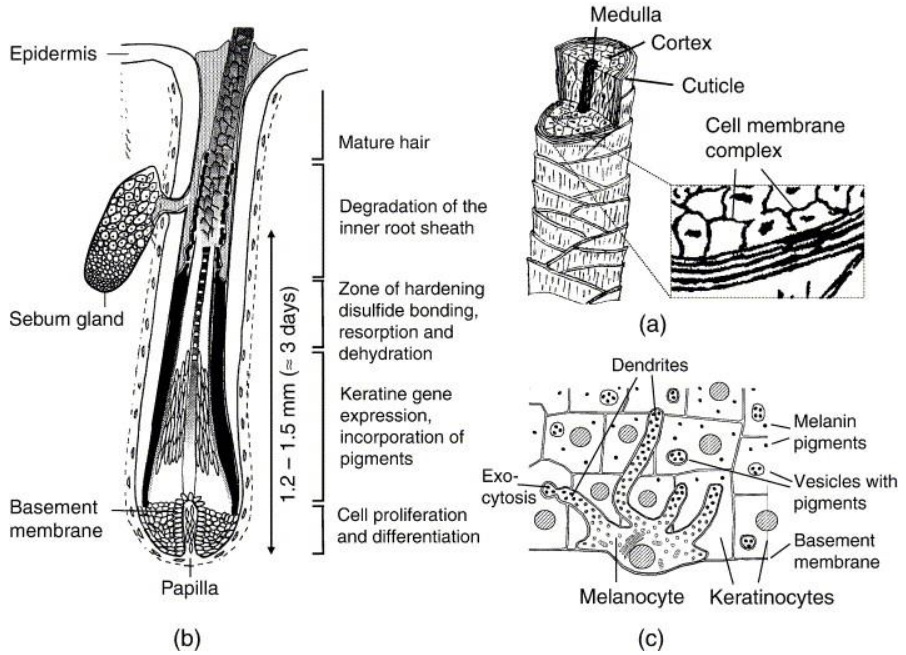


Figure 1 Structure of the human hair shaft, anatomy of the hair follicle and mechanism of melanin integration into the hair shaft. Reprinted from Clinica Chimica Acta, 370, Pragst F., Balikova M.A., State of the art in hair analysis for detection of drug and alcohol abuse, 17-49, 2006, with permission from Elsevier.

The hair follicle is the structure from which the hair shaft emerges. The origin of cell proliferation, the hair bulb, is located at the bottom of the hair follicle, ca. 3-5 mm below the surface of the skin. The hair bulb encloses the follicular dermal papilla and a single capillary loop. At the bottom of the bulb, undifferentiated cells proliferate. The cells move upwards from this region into the suprabulbar region, grow in volume, and start to elongate vertically. The hair commences keratinization and fibrils are formed. At approximately one third of the way between the tip of the dermal papilla and the skin surface, the keratinization has completed. Above the suprabulbar region, is the bulge, which is the location where the erector pili muscle attaches to the hair follicle. The short region between the bulge and the entrance to the sebaceous gland is called the isthmus. The region between the sebaceous gland and the surface of skin is called the infundibulum. This funnel-shaped structure contains the hair shaft, sebum, and apocrine sweat, in the case of an associated apocrine sweat gland. Surrounding the growing hair shaft is the inner root sheath, which extends from the base of the bulb up to the isthmus. The cells of the inner root sheath are locked with the cuticle cells of the hair shaft to firmly hold it in place. The inner root sheath grows at the same speed as the hair and guides the hair shaft in its path. Surrounding the inner root sheath is the outer root sheath, which is believed to be source of stem cells critical for the development of the hair and is the attachment point of the erector pili muscle. In contrast to the inner root sheath, the outer root sheath may remain relatively stationary.[4,8,14]

1.3. Hair Growth

A large advantage of hair analysis is that substances are incorporated and fixed in the growing hair, allowing for the long window of detection. Additionally, as substances move with the growing hair, the hair stores a chronological consumption history.[15] However, hair does not grow continually, but changes between phases of growth, rest, and finally removal with a new hair taking the place of the old hair. The hair growth phases are presented in Figure 2. The growing phase is called anagen phase and involves the construction of the hair matrix and the inner and outer root sheaths, the subsequent generation of the hair shaft, the pigmentation thereof, etc. In human scalp hair, the anagen phase has been reported to last about 1-6 years with about 80-90 % of hairs in this phase. After the anagen phase, the hair follicle is remodeled in the catagen phase. This involves among other things the involution of the lower hair follicle structures, club hair formation, and an upward movement of the dermal papilla. The duration of the catagen phase is not well described, but it is assumed to be quick and completed within a few weeks. Finally, the hair enters the telogen phase in which the hair stops growing and “rests”. The length of the telogen phase is variable with between 3-9 months for human scalp hair. About 10-20 % of scalp hair is in the telogen phase at any time. Hair can be easily mechanically removed during this stage. Plucking a hair in the telogen phase immediately starts a new cycle of hair generation. The hair cycle is a hormone driven process with each hair follicle being in this cycle individually, meaning that there is a continuous exchange of hairs on the head at any given time.[2,4,9,16]

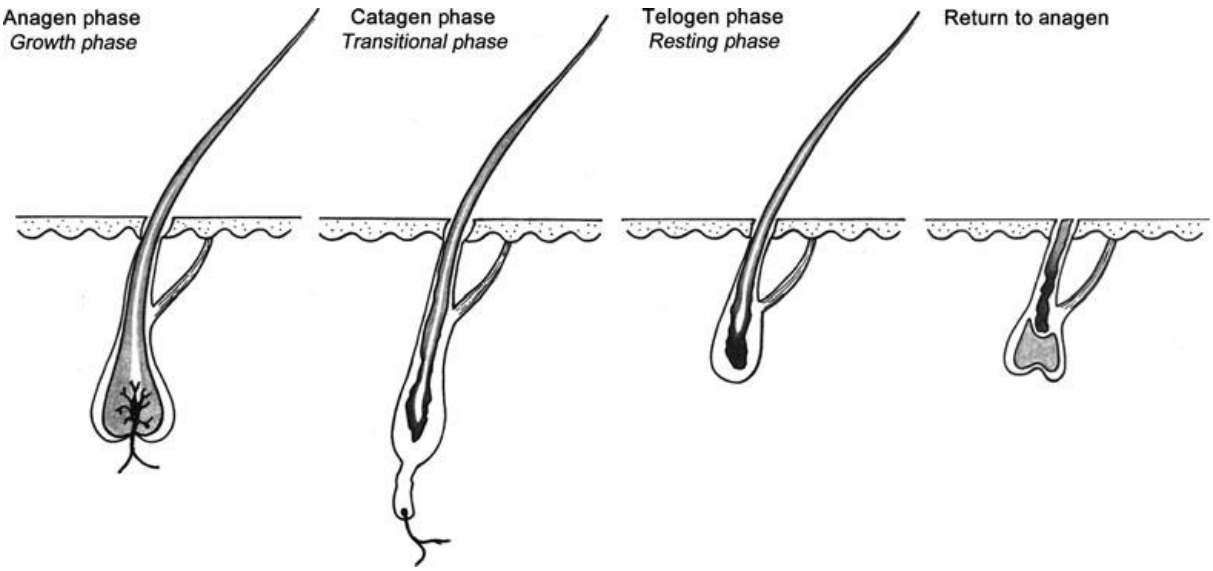


Figure 2 The phases of the human hair growth cycle. Reprinted from International Journal of Dermatology, 53, Buffoli, B. , Rinaldi, F. , Labanca, M. , Sorbellini, E. , Trink, A. , Guanziroli, E. , Rezzani, R. and Rodella, L. F., The human hair: from anatomy to physiology, 331-341, 2014, with permission from John Wiley and Sons Inc.

The growth rate of hair plays a critical part in interpreting the results of a hair analysis. The SoHT recommends using a standard growth rate of 1 cm/month for interpretation of results.[17] The range of reported growth rates is quite variable however, with rates as slow as 0.6 cm/month and as fast as 3.36 cm/month.[4,18] The growth rates as well as the proportion of hairs in the anagen and telogen phase is dependent on many factors such as age, sex and hormone levels,[19] ethnicity,[20-22] state of health, season of the year,[23] etc. This introduces a large degree of uncertainty into the chronological interpretation of a hair analysis.

1.4. Skin Anatomy, Microcirculation, Sweat Glands, and Sebum Glands

The skin is composed of three layers, namely the epidermis, the dermis, and the hypodermis or subcutaneous tissue, each of which will be described briefly. A schematic illustration of the skin anatomy is presented in Figure 3 (left). The epidermis is a thin layer of cells of around 0.05-0.1 mm thickness mainly composed of keratinocytes. Also contained in this layer are melanocytes that distribute melanin to the surrounding keratinocytes. The dermis is a supporting matrix of between 0.5 mm and 5 mm thickness. The large part of the dermis is made of collagen which provides the skin with its tensile strength. The dermis also contains a number of other proteins and structures which provide the skin with e.g. elasticity and the ability to retain water. The dermis is strongly perfused through a system of vasculature layers as presented in Figure 3 (right). The layers are the superficial or subpapillary plexus located at the edge of the epidermis from which papillary loops rise, the dermal or middle plexus which supplies e.g. the hair follicle and sweat glands with blood, and the subdermal plexus located in the subcutaneous tissue.[24-26]

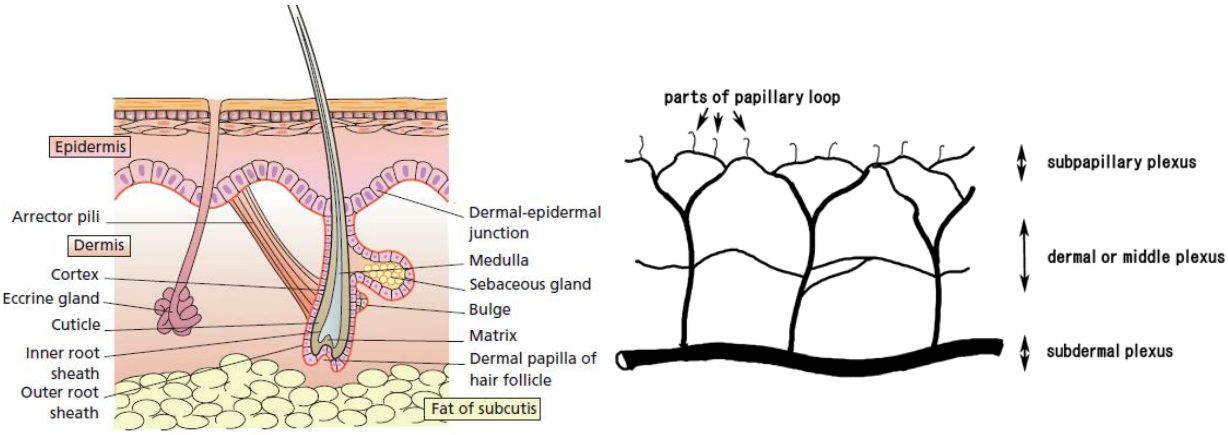


Figure 3 (left) Anatomy of the skin. Reprinted from Rook's Textbook of Dermatology, MyGrath, J.A. and Uitto, J., Anatomy and Oragnization of human Skin, 2008, with permission from John Wiley and Sons Inc (right) Illustration of the skin vascular plexuses. Reprinted from Journal of Anatomy, Imanishi, N., Kishi, K., Chang, H., Nakajima, H., & Aiso, S., Three-dimensional venous anatomy of the dermis observed using stereography, 2008, with permission from John Wiley and Sons Inc

Humans have eccrine and apocrine sweat glands. Eccrine sweat glands are located over almost the entire body including the scalp. Sweat is considered an important possible mechanism for the incorporation of substances into the hair.[2] An eccrine sweat gland is composed of a secretory coil located in the lower dermis and a duct which leads upwards and opens onto the skin surface. Eccrine sweat glands play an important part in the thermoregulation of the body. When sweat glands are activated, they pump NaCl into the secretory coil. This causes an influx of water into the coil as it now has a higher osmolality than the surrounding cells. This causes hydrostatic pressure to increase and results in the sweat gland pumping out water with maximum rates approaching 20 nL/min/gland. In the upper parts of the sweat duct, NaCl is actively transported back into the surrounding cells to limit the salt loss of the body.[27] Apart from eccrine sweat glands, apocrine glands can also be found in human skin in the genital, axillary and mammary areas. In contrast to eccrine glands, apocrine glands are directly connected with a hair follicle and sweating follows a different secretion mechanism. In apocrine glands, the secretion is accomplished by pinching off parts of the cells. The secretion is rich in lipids and plays no role in thermoregulation.[24]

Sebaceous glands are a further secretory unit of the skin. They are found all over the body with most being associated with hair follicles. They open into the upper part of the hair follicle above the bulge and are the upper border of the isthmus. Sebaceous glands consist of one or more lobules, each with a duct going to the main sebaceous duct which then goes further to the follicle. Sebaceous glands produce sebum via the holocrine rupture of sebocytes. The main components of the excreted sebum are triglycerides, wax ester, squalene, and free fatty acids.[28] The time for sebum to reach the skin surface was measured using injection of radiolabeled acetate into the forehead of four volunteers. Maximum radioactivity was measured eight days after injection.[29] Sebum is considered a further possible pathway for substance incorporation into the hair.

1.5. Incorporation of Substances into the Hair

The mechanisms of substance incorporation into hair are not fully understood. Three main routes of incorporation are usually discussed; incorporation into the growing hair via the bloodstream, incorporation via sweat or sebum, and incorporation from external sources as presented in Figure 4.[3,30,31] Incorporation via the bloodstream has typically been seen as the most important pathway and also the pathway with most interpretive value. Even for incorporation via the bloodstream, there seem to be different mechanisms, depending on the physicochemical characteristics of the substance. An analysis of the entire hair including the root in 1 mm segments 10 hours after a single zolpidem use, revealed that zolpidem is incorporated primarily at the bulb.[32] However, a second much smaller peak was found in the 2-3 mm segment, approximately corresponding to the region where sebum enters the hair follicle. In contrast, a study investigating the incorporation of EtG found that in beard hair EtG is primarily incorporated into the upper part of the hair root, instead of the bulb.[33] These results indicate that the highly lipophilic Zolpidem is incorporated differently to the highly hydrophilic EtG. A follow up study revealed that the incorporation of zolpidem is fundamentally different for colored hair than for white hair, as in white hair the peak corresponding to incorporation via the bulb was missing and only the second, smaller peak was detected.[34] An increased incorporation of basic drugs with increasing melanin content in the hair had been previously described by many researchers,[35-39] and has led to questions about racial bias in hair testing.[37,40,41] The incorporation of substances from the blood stream into the hair is assumed to be mainly affected by the lipophilicity and basicity of the substance.[42] Lipophilic drugs can enter the matrix cells of the growing hair follicle by penetrating through the cell membranes. The penetration of the membranes is only readily possible for uncharged species. The ionized species of acidic and basic substances are not able to pass though cell membranes. Therefore, the equilibrium concentrations between the sides of the membrane will be dependent on the pH on those sides. Keratinocytes and melanocytes have a lower pH than blood plasma.[43] Therefore, basic substances will tend to accumulate in hair. Metabolism of substances usually leads to more hydrophilic metabolites, as these are more readily excreted via urine. Due to the higher hydrophilicity, the metabolites might be incorporated into the hair to a lesser extent. This is one explanation for the observation that the metabolite concentrations in hair are frequently lower than what would be expected from the plasma area under the curve concentrations as compared to the parent substances.

Incorporation via sweat and sebum presents a problem for the interpretation of hair results. If substances incorporate into the hair via sweat, the chronological information of the hair may be obfuscated, as sweat can act on the entire length of the hair strand. Additionally, sweat and sebum could be transferred to the hair of a non-consumer and yield false positive results. Substances are excreted into the sweat as is demonstrated by many studies using sweat pads for abstinence testing.[44-58] There are few studies on the concentrations of substances in sweat and whether theses concentrations can lead to incorporation of relevant levels into the hair. Incorporation of substances from external sources such as powders or smoke presents a further source of false positive results.

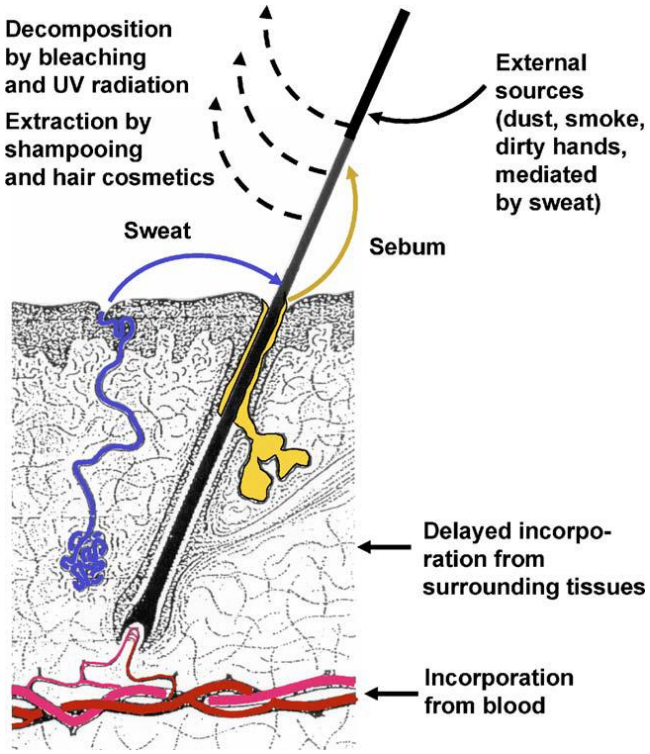


Figure 4 Proposed incorporation pathways of substances into the growing hair strand. Reprinted from Clinica Chimica Acta, 370, Pragst F., Balikova M.A., State of the art in hair analysis for detection of drug and alcohol abuse, 17-49, 2006, with permission from Elsevier

1.6. Limitations and Pitfalls in Hair Analysis

While hair as a matrix offers unique possibilities in the context of forensic work, especially the long window of detection, it also suffers from several limitations and possible pitfalls. Some of the most important pitfalls are discussed in the following sections. These pitfalls are also areas of much research and represent trends in the focus of hair analysis. New, more sensitive, methods and instruments are being employed in research to address these problems.

Hair analysis generally suffers from a poor correlation between the consumed amount of a substance and the concentrations measured in the hair. Therefore, it is not possible to determine the consumed amount of a substance from the hair concentrations. At most, a rough estimate of the consumed amount is possible and even this should be done with much caution. The underlying cause of this poor predictive value of hair results is likely the poor understanding of the exact incorporation mechanisms of substances into the hair and the contributions of the many individual factors (e.g. sex, age, BMI, genetic polymorphisms, hair color, hygienic habits, consumption style, environmental influences, hair thickness and length, etc.). Because of the difficulty in interpreting hair concentrations, many laboratories only categorize the concentrations roughly as e.g. low, mid, and high concentrations as compared with the result collective of this laboratory.

1.6.1. Variable Growth Rates and Sampling Error

One benefit of hair analysis is a chronological history of consumption starting with the present at the hair root and extending back roughly one month for every centimeter of hair. Unfortunately, the rate of hair growth varies for different people and even for hairs of the same person. A consequence of this is that the actual time period reflected by a hair sample is in the individual case usually unknown and is at best a rough estimate. Furthermore, sampling

hair strands is quite difficult and remaining stubbles on the head or unequal length of remaining hairs can cause additional errors.[59]

1.6.2. Growth Cycle, Catagen and Telogen Hair

As described above, hair grows in cycles with about 10 to 20 % of total head hair in the telogen phase. As these hairs do not grow, they do not reflect the same time period as the growing hairs around them. The longer the hair is in the telogen phase, the larger the discrepancy between this hair and the surrounding, growing hairs becomes.[59] This, together with variable growth rates of individual hair strands, causes a blurring of the chronological consumption history of the hair. If a person enters an abstinence after a time period of excessive consumption, the non-growing telogen hairs can cause hair testing to be positive in hair sample lengths that should reflect the abstinence period.[60]

1.6.3. Washout Effects

Washout effects describe the removal of incorporated substances from the hair. This can be e.g. from UV light, from shampooing and washing the hair, from exposure to chlorinated or ozonated water in e.g. swimming pools, or from cosmetic treatments.[11,12,61-75] Because of the washout effect the SoHT recommends alcohol abstinence testing in hairs of maximally 6 cm length.[76] Many studies have investigated washout effects both in vitro and for some case studies in vivo. The results are often contradictory regarding the extent or even presence of washout effects, suggesting that washout effect may be dependent on the type of substance and on the individual. Especially in the early days of hair analysis, many different methodologies for hair washing and extraction were employed. This could affect the reproducibility and transferability of the results of these studies.

1.6.4. External Contamination

One of the major issues that hair analysis is confronted with is the possibility of external contamination of the hair. In this context, external contamination describes the incorporation into the hair or adherence of substances onto the hair from external sources such as powder or smoke. External contamination can lead to a person being tested positive for a substance even though this person did not consume this substance.[77,78] This can be a problem for people who work in an environment where drug consumption is frequent such as clubs or bars, for drug enforcements officers,[79-81] or for interpreting the results from children living in an environment where drugs are being consumed.[82-84] Many different washing procedures have been proposed to deal with external contamination,[85-89] but a recent study from Cuypers et al. has shown that the washing procedures themselves can cause irreversible incorporation of external substances into the hair.[90] A different strategy for dealing with external contamination is to use metabolites to differentiate between exposure and consumption when a metabolite is available.[91-96] This strategy, while often successful, is faced with problems such as metabolites being present in the drug material or low concentrations in the hair making the analysis difficult.

1.7. The Distribution of Substances in Hair over the Scalp

A question which has so far not been addressed in this discussion, and represents the overarching topic of this thesis, is whether the location of hair sampling on the scalp, influences the concentration of substances in the hair. Numerous studies have looked at hair from different body sites and compared it to scalp hair,[97-100] but there had been only one previous publication by Dussy et al. specifically looking at differences in scalp hair depending on sampling site.[101] In this publication, Dussy showed for EtG and caffeine for three persons and for ten locations on the head that the location of hair sampling influences the concentrations with coefficients of variations between 14 – 28 % and 13 – 62 % for EtG and caffeine, respectively. However, the ten sampling locations on the head were not exactly defined and did not show a picture of the entire scalp. Therefore, statements concerning the pattern of distribution and explanations thereof were not possible from this data. Additionally, while EtG is one of the most important parameters in hair analysis, no investigation on drugs of abuse (DoA) was conducted. Finally, a sample size of three people is not enough for making general statements on the distribution patterns.

In general, hair samples are taken from the vertex posterior region of the scalp as recommended by the Society of Hair Testing (SoHT)[102] and the European Workplace Drug Testing Society (EWDTS),[103] as hair from this region has been reported to have the largest percentage of hair in the anagen phase and the highest hair growth rate.[9] Additionally, from a practical view, sampling from the vertex posterior is often easier and has a lesser impact on the haircut of the tested person. However, hair is often sampled from regions other than the vertex for reasons such as insufficient hair length at the vertex, male pattern baldness, cosmetic aspects, contamination at the vertex, etc. The SoHT proposed cut-off values are defined based on values obtained from the measurement of hair samples from the vertex posterior and should therefore strictly speaking not be applied to hair from other regions. Additionally, concentration differences in hair strands depending on the sampled region can be problematic, as it is common practice to sample two hair strands; one for the analysis and another for confirmation reanalysis in case of any doubts. If the strands are taken from different areas of the head, concentration differences can be expected, leading to ambiguous results that can cause problems when applying the SoHT cut-offs. Therefore, there is an urgent need to characterize the regional differences in scalp hair concentrations for at least the most prevalent substances EtG, common DoA and the benzodiazepines/z-substances (BZD-Z). As the mechanisms of incorporation of substances into the hair are poorly understood, it was also important to investigate the cause of the observed differences.

2. Goals and Approach

The overarching goal of the project was to characterize the shape and extent of distribution of substances in scalp hair over the entire head. From the previously mentioned study by Dussy et al. 2014, it was hypothesized that the location of sampling plays a significant role. Also, based on the differences obtained for caffeine and EtG by Dussy et al. 2014, it was hypothesized that the nature of the substance could influence the shape and extent of the distribution. The focus was placed on the most important substances for routine work at our lab; EtG, the common DoA (except THC), and the BZD-Z. Another goal was to find explanations for the suspected differences across the scalp. To approach this topic, four projects were or are currently being conducted.

Project 1 – Single-Person Distribution Study: As there was little previous information on the distribution patterns, the first goal was to confirm the findings of Dussy et al. 2014 while expanding on their work. For this, the distribution patterns of EtG and caffeine over the scalp of a single person were fully characterized. The entire scalp hair of the test subject was divided into strands of around 3x3 cm size and sampled individually, yielding around 100 strands. First and foremost, this was a pilot study to ascertain if it was worth it to keep investigating in this direction and to gain information for the planning of a more comprehensive study.

Project 2 – Combined Sample Preparation Method: As the results of the first study were very promising, the investigation of the distribution patterns was continued. The aim was to look at all the aforementioned relevant substances with a larger group of test subjects. From conducting the first project, it was apparent that the analytical workload would be overwhelming if thousands of hair strands were to be investigated for all of these substances. Therefore, a combined hair sample preparation method for EtG, DoA and BZD-Z was developed for subsequent measurement with LC-MS^3/MRM. The approach of the combined sample preparation was the separation of EtG from the DoA and BZD-Z by using their different retention characteristics on an Oasis Max SPE cartridge. While still requiring three injections to measure all parameters, the combined sample preparation could greatly reduce sample preparation time and the required amount of hair for a full analysis.

Project 3 – Distribution Patterns of EtG, DoA and BZD-Z: A study on a larger scale was planned to expand the sample size and to include cocaine, which is the most prevalent DoA beside THC in our routine population. For this, alcohol and cocaine consuming persons were enlisted and the same hair sampling as in project 1 was done. In addition to the description of the distribution patterns, possible causes for the observed differences were investigated. For this, in cooperation with the clinic for angiology at the University Hospital Basel and with the department of Sport, Exercise and Health of the University of Basel the differences in head skin perfusion and the head skin sweating rates across the scalp were investigated. A laser Doppler anemometer was used to investigate the head skin perfusion. The sweating rates were measured gravimetrically using a cycling cap lined with water absorbing pads.

Project 4 – Substance Incorporation via Sweat: From the results of project three, we suspected differences in sweating to be a major source of the concentration differences across the scalp. For sweat to be a major incorporation pathway, substances must be able to cross from the bloodstream into the sweat and then be able to go from the sweat into the hair. Most research studying the incorporation of substances from the sweat into the hair were conducted within the context of external contamination. Therefore, these studies usually focused on cocaine and employed high concentrations and unrealistic conditions for modeling the daily incorporation via sweat of a drug user. On this basis, the incorporation of our routine panel of substances into the hair from artificial sweat solutions was investigated to test the plausibility of our hypothesis that sweat is a major contributor to the concentration differences found across the head.

Project Kratom – Mitragynine and 7-Hydroxymitragynine in Hair: A study participant of project 3 declared that he consumes 3g of kratom each day. Mitragynine and 7-Hy-Mitra have recently been added to the list of scheduled substances in Switzerland. A literature search revealed no published hair concentration values or analytical methods for the major kratom alkaloid mitragynine or for the minor alkaloid 7-Hy-Mitra. Therefore, an analytical method for the quantification of mitragynine and 7-Hy-Mitra was developed and applied to all hair samples of the study participant. Additionally, the method was applied to every subsequently measured routine hair sample analyzed for DoA to estimate the prevalence of Kratom consumption in our routine population.

Project CBD – Cannabinoids in Blood and Urine after Smoking CBD Joints: Recently, hemp material containing less than 1 % THC and high amounts of CBD have become available for legal purchase in Switzerland. This material is sold with many declared purposes e.g. as tobacco replacement, as food stuffs, in cosmetics, or for vaping. Confectioned CBD cigarettes can already be bought at the supermarkets or kiosks in Switzerland. No information was available if smoking this CBD rich hemp could yield THC concentrations above the legal cut-off concentration of 1.5 ng/mL in blood (VRV Art2. Abs.2, 2.2 ng/mL with the confidence interval). Therefore, a self-experiment was conducted to determine if concentrations above this legal limit were reached. In this experiment, an employee of the institute smoked a CBD joint while blood was collected at regular intervals. Urine was collected from every spontaneous void. To see if an accumulation of THC could occur, the same person smoked two CBD joints per day for ten days. While smoking the last joint, blood and urine were again collected.

3. Publications

3.1. Project 1: Distribution Pattern of Ethyl Glucuronide and Caffeine Concentrations over the Scalp of a Single Person in a Forensic Context

About Project 1

This publication describes the first thorough investigation of the distribution patterns of EtG and caffeine in hair across the head. Large concentration differences were found and for caffeine a clear concentrations pattern was observed.

Authors: Ulf Meier, Thomas Briellmann, Eva Scheurer, Franz Dussy

Contributions of Ulf Meier:

- Involved in planning the study
- Method development for analysis
- Conducting the sample preparation, measurements and data evaluation
- Writing the article

CRediT Statement:

Ulf Meier:	Conceptualization, formal analysis, investigation, methodology, project administration, visualization, writing – original draft
Thomas Briellmann:	Conceptualization, writing – review & editing
Eva Scheurer:	Supervision, writing – review & editing
Franz Dussy:	Conceptualization, supervision, writing – review & editing

Status: Published

Research article

Drug Testing and Analysis

Received: 23 September 2016 Revised: 1 February 2017 Accepted: 7 March 2017 Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.2186

Distribution pattern of ethyl glucuronide and caffeine concentrations over the scalp of a single person in a forensic context

Ulf Meier,* Thomas Briellmann, Eva Scheurer and Franz Dussy

The distribution of analyte concentrations in hair across the scalp has not been thoroughly investigated. Differences in concentrations depending on sampling location are problematic, especially when measuring a second strand to confirm the result of the first measurement. Aiming at a better understanding of the concentration differences, the distribution of ethyl glucuronide (EtG) and caffeine concentrations in hair across the entire head of one test subject was investigated by dividing the scalp completely into regions of *ca* 2 cm × 2 cm area, yielding a total of 104 samples. For the quantification of EtG, a novel LC–MS^3/MRM method was developed and validated with a limit of detection and limit of quantification of 2 and 4 pg/mg, respectively. Large variations of the concentration across the head were found, with factors of *ca* 3.0 and 10.6 for EtG and caffeine, respectively. These differences could not be attributed to measurement error alone. The concentrations were projected onto the subject's head, and concentration patterns were identified for EtG and caffeine. When examining multiple strands from within one 2 cm × 2 cm sampling area, the strands showed similar concentrations. Segmental analysis of selected 3 cm strands showed decreasing concentrations of EtG and caffeine from proximal to distal end, possibly due to wash-out of the analytes. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: hair analysis; distribution; ethyl glucuronide; caffeine; LC–MS^3

Introduction

Many drugs and drug metabolites are incorporated into hair after consumption and remain more or less fixed in position while being mostly stable towards degradation.[1,2] In contrast to blood or urine, a hair sample allows a much larger time window of detection, which makes hair an interesting matrix for forensic questions. The long time window of detection makes it especially useful for abstinence controls. Most often this concerns drivers trying to regain their driving licence after having it suspended due to driving under the influence of alcohol or drugs of abuse, but also, for example, in child custody cases or in workplace drug testing. In Switzerland it is common practice to use a 5 cm hair strand for a surveillance window of around six months, as hair growth of around 1 cm per month is assumed with some hair being in the telogen phase.[3] For an overview of hair analysis and its applications we refer to the 2011 statement of the Society of Hair Testing (SoHT),[1] Pragst and Balikova,[2] Kintz[4] and Kintz *et al.*[5]

Ethyl glucuronide (EtG) has proven to be a suitable biomarker for alcohol consumption.[6–8] Although EtG is only a minor metabolite of ethanol and only a small part, about 0.02% of ethanol, is excreted as EtG[9,10] in urine and consequently the concentrations in hair are very low, recent improvements in analytical techniques with limits of quantification in the low pg/mg range have allowed the use of EtG to differentiate between alcohol abstinence, social consumption and chronic excessive consumption.[3,7,11] A GC-NCI-MS/MS method with a very low limit of quantification of 0.2 pg/mg has recently been reported.[12] As stated in the SoHT consensus[11] a concentration higher than 7 pg/mg up to 30 pg/mg in hair segments of 3–6 cm measured from the skin strongly suggests moderate alcohol consumption, while a lower concentration does not contradict a self-reported abstinence. Additionally, if the concentration of EtG in this segment exceeds 30 pg/mg, chronic excessive alcohol consumption corresponding to a daily average of 60 g or more of pure ethanol over several months is very likely.

Although hair analysis has been employed for more than 35 years, with Baumgartner *et al.* determining opiate content of hair by radioimmunoassay in 1979,[13] there are still many fundamental questions which have not yet been fully answered. One such question is the distribution of EtG and other drugs and their metabolites in head hair. Dussy *et al.* demonstrated that hair strands taken from different areas of the head at the same time showed different concentrations for EtG and for caffeine, which might serve as a good model substance for basic drugs of abuse.[14] Analyte concentration differences in hair strands depending on the sampled region can be problematic for multiple reasons. It is common practice to take two hair strands during sampling; one is used for the analysis while the second is stored and can be used for reanalysis in the case of any doubt. If these two strands are taken from different areas of the head their analyte concentrations might differ. This can lead to ambiguous results and problems for decision making when applied to the limits recommended by the SoHT, and can have serious consequences for the person being tested. Additionally, when doing follow-up investigations to monitor

* *Correspondence to: Ulf Meier, Institute of Legal Medicine of the University of Basel, Forensic Chemistry and Toxicology, Basel, Switzerland. E-mail: ulf.meier@bs.ch*

Institute of Legal Medicine of the University of Basel, Forensic Chemistry and Toxicology, Pestalozzistrasse 22, CH-4056 Basel, Switzerland

consumption, taking hair strands from different areas on the scalp could lead to false interpretation of the consumption development of the tested person. The SoHT recommends strands to be taken from the vertex region of the scalp.[11] This is, however, not always possible. Additionally, the recommendation is based on the hair growth rates being mostly uniform in this region and not on data about drug deposition into hair in this area. The problem is exacerbated by the not fully understood incorporation mechanism of drugs, with blood and sweat being suggested as main pathways for EtG.[2] Schräder *et al.* recently found evidence for incorporation from blood being the main pathway.[15] For a good interpretation of results, it is important to gain an understanding of the maximum variations that can occur, and if possible to identify a distribution pattern for the analytes over the head. For this purpose, all head hair of a volunteer, yielding a total of 104 strands, was analysed for EtG and caffeine to map the concentrations over the entire scalp. For this, a novel LC–MS^3/MRM method for EtG and a LC–MS/MS method for caffeine with a combined extraction procedure were developed and validated.

Materials and methods

Chemicals

The EtG standard used in the calibration row and the deuterated internal standard ethyl glucuronide (EtG-D5) were purchased from Lipomed (Arlesheim, Switzerland). The caffeine standard used in the calibration row and the deuterated internal caffeine (caffeine-D9) standard were purchased from Sigma-Aldrich (Buchs, Switzerland). The LC-MS solvents water, acetonitrile and methanol were all obtained in analytical grade purity from Machery-Nagel AG (Oensingen, Switzerland). Formic acid puriss p.a. (98%) was obtained from Sigma-Aldrich (Buchs, Switzerland). 2-Propanol (≥99.5%) and acetone (≥99.5%) used for washing the hair was purchased from Roth (Arlesheim, Switzerland). Deionized water used for washing the hair was produced in house. Ammonium hydroxide solution (25%) used for making 1% ammonia LC-MS solvent was purchased from Sigma-Aldrich (Buchs, Switzerland). The cartridge used for solid-phase extraction (SPE) was an anion exchange mixed mode Oasis Max 60 mg, 3 mL cartridge purchased from Waters (Daettwil, Switzerland).

Study protocol

The scalp of one volunteer, a 46-year-old male, who declared himself a social drinker (ethanol daily intake (EDI) ≈ 35 g), was divided into quadratic areas of roughly uniform size (2 cm × 2 cm). From each of these areas all hair was collected close to the scalp, giving a total of 104 separate strands collected at the same time. The numbering of the sampled areas is shown in Figure 1. The test person had cosmetically untreated hair of brown colour with a few grey hairs. The weight of each strand was between 50 and 350 mg

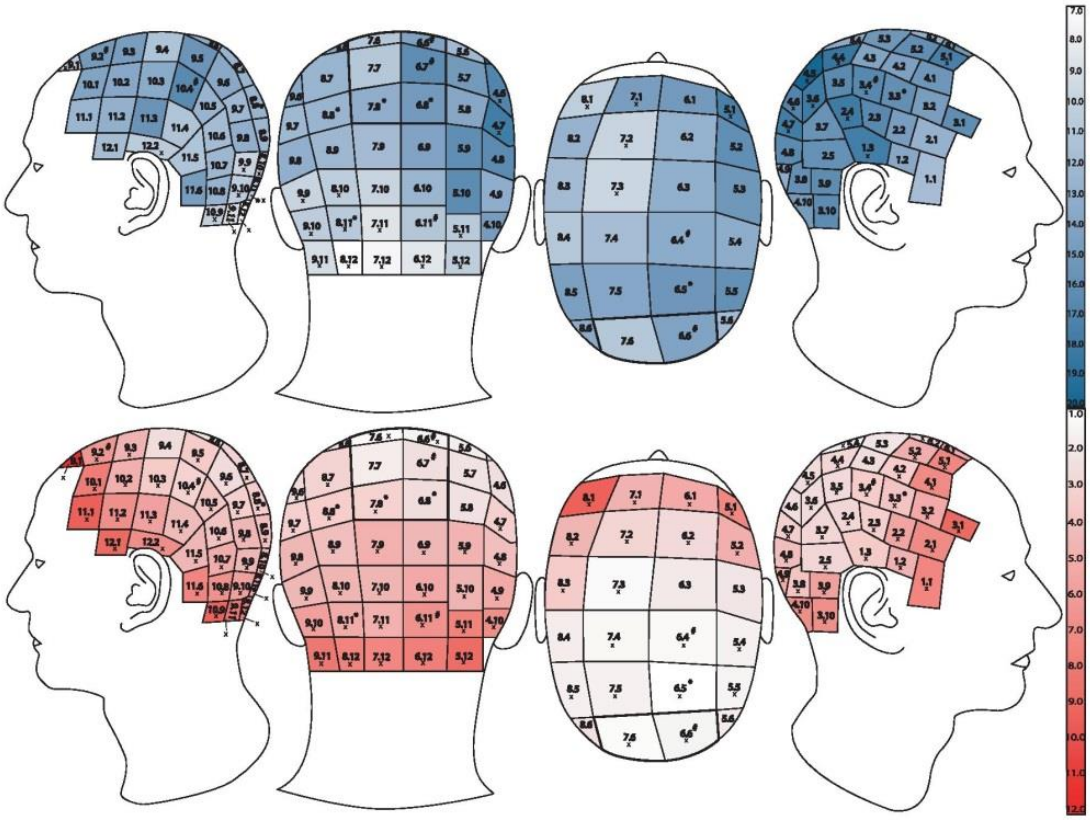


Figure 1. Projections of the measured concentrations of EtG (top) and caffeine (bottom) onto the head of the test subject. Areas marked with an asterisk (*) show sampling regions for which the hair strand was separated into multiple smaller strands to test for homogeneity. Areas marked with a hashtag (#) show sampling regions for which a segmentation of the strand was done. The bold outline of the six cells 6.6, 6.7, 6.8, 7.6, 7.7, 7.8 shows the vertex posterior region. Sampling regions that show significantly different concentrations from the mean of the vertex posterior are marked with an 'x'. [Colour figure can be viewed at wileyonlinelibrary.com]

after being cut to a uniform length of 0–3 cm. An aliquot of hair (*ca* 50 mg) was taken from each strand and homogenized. Every sample was then analysed for EtG, and for caffeine as a model substance for basic drugs of abuse. For seven strands (3.4, 6.4, 6.6, 6.7, 6.11, 9.2, 10.4) the proximal end was aligned and the 3 cm strand cut into three 1 cm segments and each segment analysed for EtG and caffeine. The corresponding areas are marked with a hashtag (#) in Figure 1. Six strands (3.3, 6.5, 6.8, 7.8, 8.8, 8.11) were divided into four smaller strands to test for the homogeneity within one sample area. The corresponding areas are marked with an asterisk (*) in Figure 1. For strand 8.11 there was only enough hair to be divided into three strands. The smaller strands were made by dividing the sampled strand and were not sampled separately and therefore do not correspond to the use of a smaller sampling grid.

Sample preparation

For the analysis of both EtG and caffeine, a combined sample preparation and extraction was done. The strands were washed with 3 mL of water, followed by 3 mL of 2-propanol and lastly 3 mL of acetone. The strands were either allowed to dry by leaving them overnight or placed under a soft stream of nitrogen for *ca* 15 minutes. The washed and dried hair strands were cut using scissors into snippets of around 1 mm. Approximately 20 mg of the hair snippets was then pulverized using an MM 200 ball mill (Retsch, Schieritz & Hauenstein, Arlesheim, Switzerland) operated with a frequency of 30 Hz for 7 min. EtG-D5 (500 pg) and caffeine-D9 (100 ng) were added to the ground hair. The hair was then extracted for between 1 and 1.5 h using 1.5 mL of water. The validation of a GC–MS method adapted from Yegles *et al.*[16] for EtG determination at our laboratory has shown that extraction time variations in this range do not influence the extraction efficiency. The sample was centrifuged for 10 min. The supernatant was added to the Oasis Max SPE cartridge which had been conditioned with 2 mL of methanol followed by 2 mL of water. After addition of the sample, the cartridge was washed using 1 mL of water. Caffeine was eluted using 2 mL of methanol. The cartridge was dried for 10 min by sucking air through the cartridge by a vacuum pump. EtG was then eluted using 2 mL of methanol with 2% formic acid. In a preceding experiment all SPE fractions were collected and examined for both caffeine and EtG. Both substances were only found in their corresponding fraction. The SPE procedure was adapted from procedures successfully used by different laboratories for EtG determination.[17–19] Both the caffeine- and EtG-containing extracts were dried separately under a gentle nitrogen stream. The caffeine extract was reconstituted in 1 mL of water, while the EtG extract was reconstituted in 50 μL of water.

EtG LC-MS^3/MRM method

The LC method was adapted from Binz *et al.*[17] Briefly, EtG analysis was done with an Ultimate 3000 high performance liquid chromatograph (Dionex, Reinach, Switzerland) coupled to a 5500 QTrap triple quadrupole mass spectrometer (Sciex, Brugg, Switzerland) operated in negative electrospray ionization mode (ESI). A Hypercarb, 100 × 2.1 mm, 3 μm column (Thermo Fischer, Reinach, Switzerland) protected by a 10 × 2.1 mm, 3 μm pre-column (Thermo Fischer, Reinach, Switzerland) with a flow rate of 400 μL/min was used with the following LC programme: 1% fresh ammonia solution was used for the gradient programme with 100% (1 min hold) to 90% at 4 min, 10% at 8 min (2 min hold), 0% at 10.2 min and 100% at 12 min (hold 3 min) adjusted with acetonitrile to 100%. The EtG was measured with injection of 20 μL with a combined multiple reaction monitoring (MRM) and MS^3 method operating in negative ESI mode. The instrument was operated with an ion-spray voltage of −4500 kV, a source temperature (TEM) of 600°C, a curtain gas (CUR) of 40 psi nitrogen and nebulizer (GS2) and heating (GS1) gas of 80 and 85 psi, respectively. The collision associated dissociation gas (CAD) was set to 11. The mass transitions used were MS^3 (221.1/113.0/85.1) and MRM (221.1/75.0; 221.1/55.0) for EtG and MRM (226.0/75.0; 226.0/55.0) for EtG-D5. The instrument parameters are described in Table SI.1. A total cycle time of 0.41 s resulted for the method. Data analysis was carried out using MultiQuant software (version 3.0.2, Sciex, Brugg, Switzerland). The total runtime of the method was 16 min with EtG eluting at 3.7 min.

During method development loss of intensity was observed after measurement of *ca* 30–40 samples. Flushing the capillary with a high flow rate of methanol (3 mL/min) for 10 min prior to a new series prevented the loss of intensity. If this is not sufficient, an acidic methanol solution (30% formic acid) can be used. Intensity loss was observed if the ammonia solution used was several days old. Therefore, it is suggested to regularly make a new ammonia solution.

EtG method validation

The linearity of the method was tested by measuring calibration rows in child blank hair with seven calibration levels (5, 20, 40, 60, 80, 100, 120 pg/mg) corresponding to the range of interest for hair analysis in six replicates for each concentration level. A calibration row was additionally measured in water. The calibration curve was estimated using least-squares regression analysis. Linearity was estimated with the squared correlation coefficient (R^2). The suitability of a linear model was verified by Mandel's linearity test (99% significance level). The calibration was examined for homoscedasticity using an F-test (95% significance level) between the lowest and highest concentrations.

To evaluate the selectivity of the method, blank hair extracts of two children, both below the age of eight, as well as ten hair samples of self-proclaimed teetotallers were analysed.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated using EtG-spiked child blank hair samples and requiring a signal to noise ratio of at least 3:1 and 10:1, respectively, as well as the measured concentration being within ±20% of the expected concentration for the LOQ. Noise was measured ±0.05 min around the peak.

The accuracy and precision of the method were evaluated using four international proficiency test samples (SoHT; the German-speaking society of toxicology and forensic chemistry GTFCh) and a quality control (QC) sample measured multiple times ($n = 20$) over a duration of three months by different operators, respectively. The QC sample consisted of a homogenized hair pool, from which a sample was analysed in every measurement series. The four international proficiency test samples were measured with the new LC-MS^3/MRM method, and the results were compared with a validated and accredited GC–MS method of Dussy *et al.*[14] adapted from Yegles *et al.*[16] and Martins Ferreira *et al.*[20] at our laboratory as well as with the mean results of all participating laboratories.

Matrix suppression or enhancement was estimated by injecting a blind hair extract into the LC while directly infusing EtG into the MS via syringe during the entire run time. This results in a constant signal for EtG during the entire acquisition with matrix constituents potentially causing an enhancement (increased signal intensity) or suppression (decreased signal intensity) at their retention time.

As the sample preparation and SPE procedure were identical to the validated GC–MS method for EtG,[14] validations concerning these parts of the method were not repeated.

Caffeine LC-MRM method

The caffeine method was adapted from Dussy *et al.*[14] All instrument settings were identical to the published method. The extraction was changed as described above. Caffeine analysis was done using an Ultimate 3000 high performance liquid chromatograph (Dionex, Reinach, Switzerland) coupled to a 4000 QTrap triple quadrupole mass spectrometer (Sciex, Brugg, Switzerland) operated in atmospheric-pressure chemical ionization (APCI) mode. A Phenomenex Kinetex 50 × 2.1 mm, 2.6 μm column (Brechbühler, Schlieren, Switzerland), protected by a Phenomenex KrudKatcher Ultra 0.5 μm 316 stainless steel depth filter pre-column (Brechbühler, Schlieren, Switzerland) with a flow rate of 500 μL/min was used with the following LC programme: formate buffer (10 mM ammonium formate set to pH 3.4 with formic acid) was used for the gradient programme with 99.5% (1.5 min hold) to 90% at 2 min and 40% at 12 min adjusted to 100% with methanol.

Caffeine was measured with injection of 5 μL with an MRM method operating in positive APCI mode. The instrument was operated with an ion-source temperature of 400°C, a curtain gas (CUR) of 25 psi and a nebulizer gas of 30 psi. The collision associated dissociation gas (CAD) was set to 6. The transitions used were MRM (195.2/138.1; 195.2/110.1; 195.2/83.0) for caffeine and MRM (204.2/144.0; 204.2/116.2; 204.2/126.0) for caffeine-D9. The instrument parameters are described in Table SI.2. The total cycle time was 0.64 s. Data analysis was carried out using MultiQuant software (version 3.0.2, Sciex, Brugg, Switzerland). The total runtime of the method was 21 min with caffeine eluting at 5.0 min.

Caffeine method validation

As only relative caffeine amounts in different hair samples were of interest and not the absolute values, only a partial validation of the caffeine method was performed. Linearity was tested with a calibration row with eight points (0.5, 1, 3, 5, 7, 9, 11, 13 ng/mg) in blank hair matrix (child hair, under eight years old) with each level measured in six replicates. Additionally, a calibration row was measured in water. Linearity was estimated using the squared correlation coefficient (R^2). The suitability of a linear model was verified by Mandel's linearity test (99% significance level). The calibration was examined for homoscedasticity using an F-test (95% significance level) between the lowest and highest concentrations. The selectivity of the method was not tested, as no total caffeine abstainers could be found. Accuracy was evaluated by measuring five blank hair samples spiked with 2 ng/mg caffeine. The LOQ was determined from the calibration row, with the requirements for the LOQ being a signal to noise ratio of at least 10 and the measured concentration being within ±20% of the expected concentration. Noise was measured ±0.05 min around the peak. The LOD was not determined. To evaluate the precision of the method a QC sample was run with every series. The QC sample consisted of the caffeine-containing fraction obtained from the EtG QC hair sample.

Data analysis

All calculations and statistics were processed using Microsoft Excel 2010 (Redmond, WA, USA). The expanded measurement uncertainty for the method was calculated by expanding the coefficient of variation (CV) by a coverage factor of 2 for a confidence level of approximately 95%. Levene's test for homogeneity of variances was done using the Real Statistics resource pack (www.real-statistics.com) for Excel. A value of $p < 0.05$ was considered significant. To evaluate if two values were significantly different, the 95% confidence interval (CI) of the difference was used, calculated using the expanded measurement uncertainty of the method. If the CI of the difference of two values contained zero, the difference was considered not significant and vice versa. All graphs were made using GraphPad Prism 5 (La Jolla, CA, USA).

Results and discussion

EtG method validation

Both the calibrations in water and in blank hair extract were linear over the whole range up to 120 pg/mg with R^2 values of at least 0.99 and identical slopes. Mandel's linearity test confirmed the appropriateness of the linear model. Homoscedasticity was not given over the whole range, which is not unexpected for a concentration range spanning a factor of 20. Using a weighing factor of $1/x$, homoscedasticity of the relative errors was achieved over the whole range. The applicability of the calibration was verified by measurement of proficiency test samples. Results are displayed in Table SI.3.

No EtG was found in the blank child hair samples or the samples of the self-proclaimed teetotallers. The LOD and LOQ values determined from spiked child hair extracts were 2 and 4 pg/mg, respectively. The precision after 20 measurements of the QC sample during 14 weeks yielded a mean of 11.7 pg/mg, a CV of 7.8% and a maximum and minimum of 13.3 and 10.1 pg/mg, respectively. This gives an expanded measurement uncertainty of 15.6% for the method. Results of the proficiency test samples are displayed in Table SI.3. The measurement of the four proficiency test samples using the new LC-MS^3/MRM method had a mean bias of +3.4% compared to the results of all participating laboratories. The results of the new LC-MS^3/MRM method were comparable to those of the validated GC–MS method.

While the LOD/LOQ of this new MS^3 method are not superior to those of other published methods,[12,17] the use of a second fragmentation step and collection in the ion trap allowed the circumvention of interferences otherwise seen for the used LC method. The transitions MRM (221/85 and 221/75) from the published method[17] showed large interferences in the low pg/mg range (<10 pg/mg) at our laboratory leading to a severe overestimation of the EtG present for some hair samples. When using the transition (221/113/85) this interference was not seen and samples in this low concentration range could be more securely quantified. Therefore, the transitions (221/113/85 and 221/55) were used as quantifiers while the transition (221/75) was used only as qualifier. Figure 2 shows the chromatogram for a hair sample (strand 8.12) with a measured EtG concentration of 6.8 pg/mg.

A matrix suppression effect was seen at a retention time of about 1 min. However, this is not in the relevant retention time window of 3.5 to 3.8 min. No other matrix suppression effects were observed.

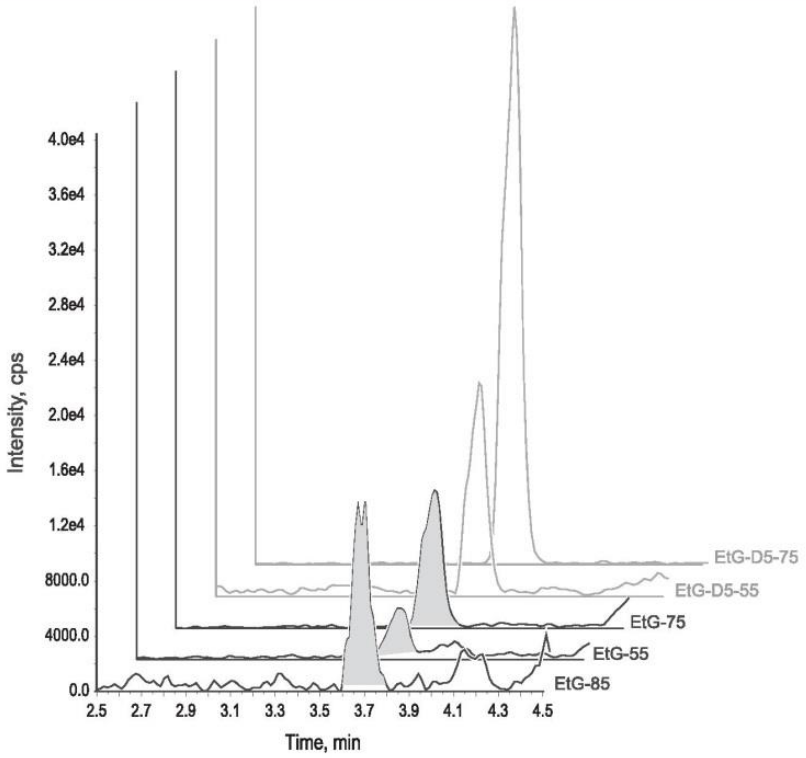


Figure 2. Chromatogram of a real hair sample (strand 8.12) containing 6.8 pg/mg EtG. (Front to back) Shown are the transitions (221/113/85), (221/55), (221/75), (226/55), (226/75). The ISTD transitions are displayed in grey.

Caffeine method validation

Both the calibrations in water as well as in blank hair extract were linear over the range 1 to 13 ng/mg with R^2 values of at least 0.99 and showed identical slopes. At low concentrations some interfering signals were observed. Thus the 0.5 ng/mg calibration point was not used and the LOQ was set to 1 ng/mg. Mandel's test confirmed the use of a linear model. Homoscedasticity was not given over the tested range. However, using a weighing factor of $1/x$ homoscedasticity of the relative errors was given over the calibration range. Five blank child hair samples were spiked with 2 ng/mg caffeine each and measurements yielded an average value of 2.4 ng/mg and a CV of 3.8%. The average value being above 2 ng/mg can be explained by the blank hair sample containing some caffeine (<0.5 ng/mg). The LOQ was set to 1 ng/mg as there were some interfering signals causing signal to noise ratio to be below 10 for the 0.5 ng/mg calibration point. The QC sample was measured with every series (n = 16) and yielded a mean of 1.7 ng/mg, a CV of 6.7%, with a minimum and maximum of 1.5 and 1.9 ng/mg, respectively. This results in an expanded measurement uncertainty of 13.4%.

Distribution pattern

EtG and caffeine were analysed in all 104 head hair strands. The results were projected onto the head of the volunteer as shown in Figure 1. Exact values are shown in Table SI.4.

The measurement of the 104 strands resulted in a mean EtG value of 13.7 pg/mg and a maximum and minimum of 20.2 and 6.8 pg/mg, respectively (strands 4.5 and 8.12), and a CV of 19.5%. Thus, a factor of 3.0 between the maximum and minimum concentration was found. The concentration values and the distribution density are shown in Figure 3a.

To see if the distribution could be attributed to the measurement error of the method, the null hypothesis of homogeneous variances for the distribution of the 104 samples and the QC sample was tested with Levene's test based on the median of the groups. The variance found for the QC sample stems only from measurement error, as all QC samples were taken from the same pool. A non-significant difference in variance shows that the found differences across the head can be attributed solely to measurement error while a significant difference means the variation within the 104 samples cannot be attributed solely to measurement error. Applying Levene's test yielded a p-value of 2.9×10^{-5}, causing the null hypothesis of homogeneous variances to be discarded at the 95% confidence level. This shows that the found variation cannot be attributed only to the measurement error and that the distribution of EtG is significantly different among different regions across the scalp. The significance of each measurement against the mean of the vertex posterior region (13.6 pg/mg) was tested and is shown as a grey band in Figure 3a. For EtG, 74% (77 measurements, all other regions marked with an 'x' in Figure 1) were not significantly different from the mean of the vertex posterior region and the mean of the vertex posterior was very close the mean of all measurements. This shows that, for this particular head, a randomly taken sample would have a high probability of having a concentration close to the vertex posterior region. The distribution density follows a Gaussian like curve with similar frequencies of the intervals above and below the middle of the whole concentration range.

The measurements of the 104 strands yielded a mean caffeine value of 4.6 ng/mg, with a maximum and minimum of 12.0 and 1.1 ng/mg, respectively (strands 9.1 and 6.5), and a CV of 49.4%. This

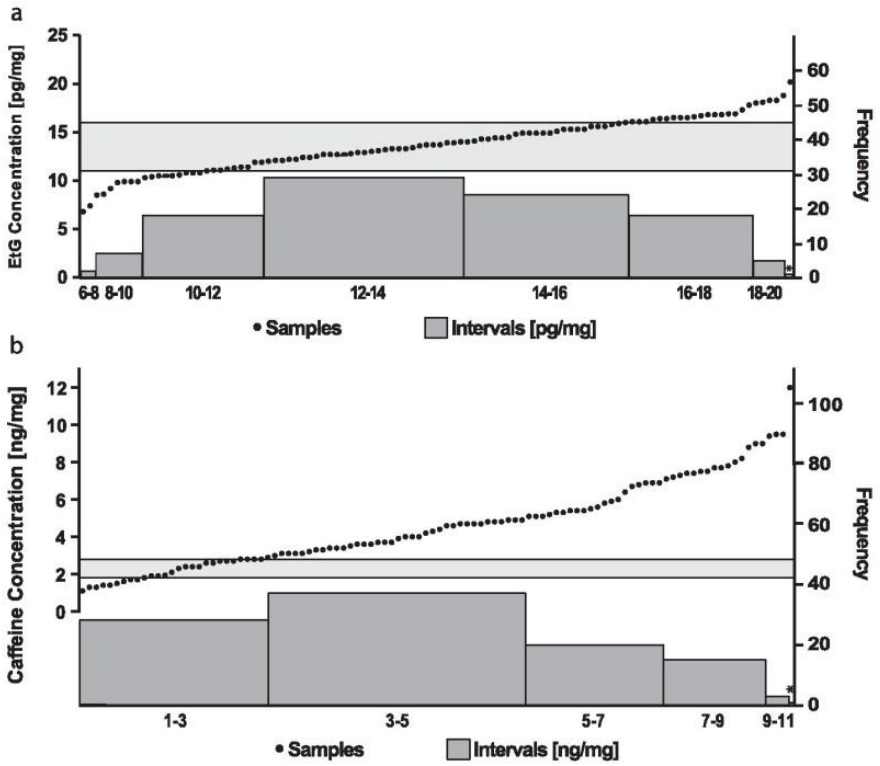


Figure 3. Distribution of all obtained results for EtG (a) and caffeine (b). Dots show the values of the 104 samples (left axis). Columns show the density distribution, with the height of the column showing the number of samples found within the marked interval (right axis) and the width showing the corresponding points. The grey band shows results which are not significantly different from the mean of the vertex posterior region at the 95 % confidence level. The asterisk (*) show the intervals 20–22 pg/mg for EtG (a) and 11–13 ng/mg for caffeine (b).

results in a factor of 10.6 between the maximum and minimum concentration. The concentration values and the distribution density are shown in Figure 3b.

Using Levene's test as above, a p-value of 7.8×10^{-7} was obtained, again causing the null hypothesis of homogeneous variances to be discarded and showing that the variations are significantly different and cannot be attributed only to measurement error. The significance of each measurement against the mean of the vertex posterior region (2.2 ng/mg) was tested and is shown as a grey band. For caffeine, 13% (13 measurements, all other regions marked with an 'x' in Figure 1) were not significantly different from the mean of the vertex posterior region. In contrast to EtG, taking a random sample would have a high likelihood of showing a result that is significantly different from the mean of the vertex posterior. This is expected as the extent of concentration variation for caffeine is larger, while the measurement uncertainty is slightly lower. It should be noted that the vertex posterior region was not very homogeneous itself with increasing concentrations towards the neck and there was a factor of 2.5 between the lowest and highest sample within this region (strands 7.6 and 7.8, respectively). This expresses itself as three samples from the vertex posterior being considered significantly different from the mean of the vertex posterior when using the described test. The distribution density for caffeine shows higher frequencies for concentrations below the middle of the whole concentration range with a long tail towards the higher concentrations.

There is a clear difference between the distribution of EtG and caffeine in the tested samples. The factor between the maximum and minimum concentrations is about threefold higher for caffeine than EtG. The distribution pattern is also quite different for the two analytes as shown in Figure 1. Caffeine generally shows higher concentrations on the edges of the haircut, with the concentrations decreasing with proximity to the scalp centre. In contrast, EtG shows the highest concentration above the ears and towards the front of the head, while the lowest concentrations were seen at the back of the neck. Additionally, the right side of the head tends to show higher concentrations than the left side for EtG. As perfusion is assumed to be symmetrical for both head sides, a difference in the distribution of sweat could be a possible reason, resulting for example from the preferred sleeping position of the test subject. It is interesting to note that one hair sample even showed a concentration below the cut-off of 7 pg/mg used to assert abstinence, while the rest showed concentrations typical for moderate intake, in line with the self-reported alcohol consumption. No value, however, exceeded the 30 pg/mg cut-off proposed by the SoHT[11] for chronic excessive drinking. Generally, neighbouring sample areas showed similar concentrations. For each sampled area a maximum relative deviation to all neighbouring areas was calculated. The highest relative deviation for one area to its neighbouring areas was 66% (strand 5.6 to 4.5) for EtG and 262% (strand 7.3 to 8.2) for caffeine. These deviations are far below the deviations seen for EtG and caffeine across the whole head. This supports the observation based on Figure 1 that the concentrations follow a rather continuous distribution and are not erratically distributed. The concentration of each region was compared with the mean of the vertex posterior, as sampling from the vertex posterior is the current recommendation of the SoHT,[11] due to fast and regular hair growth in this region.[21] However, further biological relevance could not be attributed to the vertex posterior based on the presented results. For caffeine, the vertex does not reflect the

minimum, mean or maximum concentration found on the head. Additionally, a high variability of caffeine concentrations was even found within the vertex. For EtG, the mean concentration of the vertex is similar to the mean across the head. However, we believe this to be by chance and not to be generally true.

There are three suggested main pathways for substance incorporation into hair: through the bloodstream in the papilla up to the isthmus, through incorporation by passive diffusion from sebum and sweat, and finally from external contamination. Substance incorporation can be affected by a number of factors, e.g. hair colour, hair treatment, head or body hair and lipophilicity or hydrophilicity of the substance itself.[2,22,23] It has recently been shown that incorporation of EtG through the bloodstream is the dominant pathway in beard hair.[15] However, it is possible that for longer hair, like the head hair examined in this study, sweat plays a larger role than for daily shaved beard hair. The difference in the concentration distribution patterns between EtG and caffeine suggest that they might be caused by different incorporation mechanisms of the analytes into the hair. A combination of pathways is most likely, and from these data it is not possible to determine if different levels of perfusion of the hair follicle or different amounts of transpiration are responsible for the found non-uniformity. The volunteer only had very few grey hairs that were equally distributed over the head and they will therefore not meaningfully contribute to distribution differences. An external contamination for EtG is unlikely, as it is a metabolite only formed inside the body. Caffeine incorporation by external contamination is possible, e.g. from contact of hands with coffee and afterwards with the head hair, but it seems unlikely that this is a major contribution to the concentrations.

Intra-sample area homogeneity

The results of the six strands divided into four smaller strands are displayed in Figure 4. The exact results can be found in Table SI.5. When applying the expanded measurement uncertainty to the mean of the divided strand results, all results lie within the interval for EtG. For EtG the CV values obtained for the measurements within one strand were between 3.8 and 10.6% (strands 7.8 and 8.11) with a mean CV of 7%. For caffeine two values from both strands 6.8 and 7.8 were outside the interval. For caffeine the CV values were between 3.6 and 17.4% (strands 8.8 and 6.8) with a mean CV of 10.0%.

These results show that the sample obtained from one 2 cm × 2 cm area was fairly homogeneous. Some inhomogeneity was expected as there was most likely a concentration gradient within the sampled area resulting from the gradient running across the whole head. As the gradients observed were more pronounced for caffeine, more inhomogeneity was expected for caffeine than for EtG. However, the values obtained for one area lie much closer together than the values obtained for the complete sample set of 104 strands. This supports that the non-uniformity over the entire head was not caused by small clusters of strongly varying concentrations, but is most likely caused by larger concentration gradients running over the head.

Strand segmentation

The results for the segmentation experiments are displayed in Figure 5. The exact results can be found in Table SI.6. For both EtG and caffeine, a trend towards lower concentrations at the distal end was observed. The concentration ratio from the proximal to the distal end ranged from 1.5 to 2.7 (strands 3.4 and 6.4) for EtG with a mean of 2.1 (CV = 20%). For caffeine the ratio ranged from 1.1 to 3.2 (strands 6.11 and 6.7) with a mean ratio of 1.9 (CV = 35%). It should be mentioned here that sampling a 2 cm × 2 cm area is difficult and a higher offset in distance of cutting from the skin surface should be expected than when sampling smaller strands.

The observation of decreasing concentrations from the proximal to the distal end could be attributed either to a change in the consumption over time or to a wash-out effect for EtG and caffeine causing a hair-age dependent elimination from the hair. Here the exact consumption behaviour of EtG and caffeine during the corresponding three months was not documented. The test subject estimated his EDI to be around 35 g. Therefore, it cannot be completely ruled out that the observed decrease in concentration towards the distal end can be explained partially by the consumption behaviour. There are many factors which could influence the presence or extent of wash-out effects such as frequency of showering or shampooing habits. Even the time of year could have an influence, e.g. amount of UV light, more sweating, etc. The hairs of the test subject correspond roughly to a time period of the three months of August, September and October. The washing behaviour was not recorded during this time. However, the test subject estimated that the hair was washed

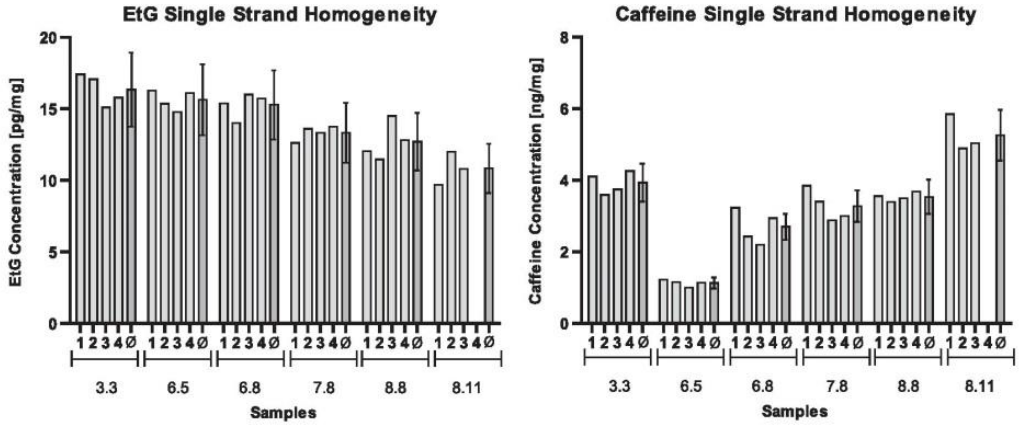


Figure 4. Results obtained for the intra-sample area smaller strands (light grey) for EtG (left) and caffeine (right) and the average (dark grey) of the results for one sampled area. The expanded measurement uncertainty of the method was applied to the mean of the single strand results. The name of the sampled areas is shown below the bars.

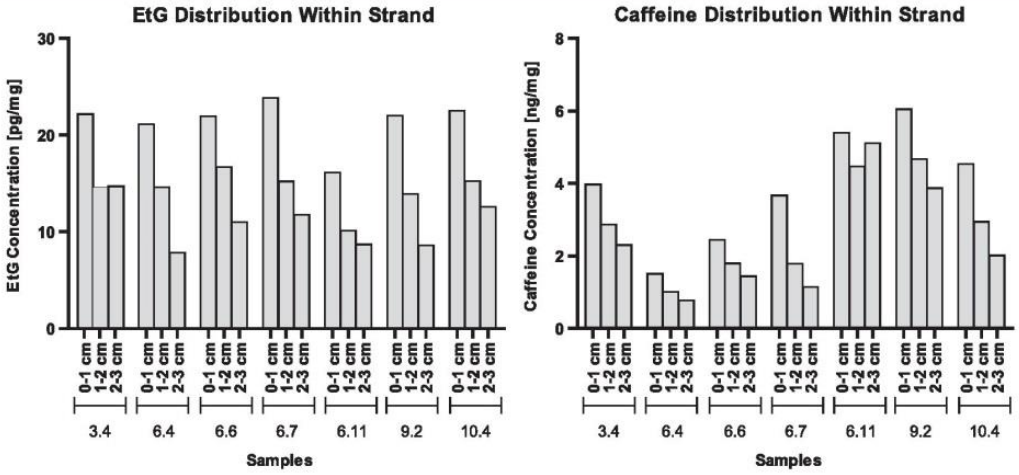


Figure 5. Results obtained for the strand segmentation experiment for EtG (left) and caffeine (right). The bars show the proximal segment from 0–1 cm, the middle segment from 1–2 cm and the distal segment from 2–3 cm (left to right bars). The name of the sampled areas is shown below the bars.

roughly every second day using shampoo. The presence of a wash-out effect for EtG is controversially discussed within the literature with evidence existing for and against such an effect. Tsanaclis *et al.* found a decrease of EtG from proximal to distal end within 3 cm strands cut into three 1 cm segments for a very heterogeneous group of samples with no knowledge of drinking or hair washing behaviour.[24] The ratio of mean concentrations in the proximal to the distal end was *ca* 2.2, which is nearly identical to the ratio found in the present study. More recently, Agius *et al.* found no significant decrease of the EtG concentration in hair for several strands longer than 3 cm, but found an increase of positive EtG results when looking at a larger population of strands of less than 3 cm length compared to longer strands,[25] which was attributed to incorporation from sweat due to recent alcohol consumption. In summary, care should be taken when interpreting consumption trends, and a wash-out effect should be considered. Additionally, the SoHT cut-off should not be strictly applied to strands shorter than 3 cm, as is also stated in the consensus.

Although the exact consumption behaviour of caffeine was not recorded, the test subject reported a very regular coffee and tea drinking behaviour. Therefore, the observed caffeine concentration decrease towards the distal end can most likely not be explained by the consumption behaviour and is rather caused by a wash-out of caffeine. No literature pertaining to a wash-out effect of caffeine was found. However, as caffeine is quite hydrophilic, a wash-out effect seems plausible.

Conclusion

When examining the concentration of EtG and caffeine in head hair across the whole scalp of one volunteer, concentration differences between different head areas were observed. These differences were larger than the measurement error expected from the method. When examining the maximum relative deviation of one sample area to its direct neighbours, differences much lower than the differences found across the whole head were seen. This is evidence for concentration gradients running across the head.

A large concentration variation across the head for an analyte, as was found here for EtG with a factor of three, constitutes a problem for the interpretation of the results. If a measurement is questioned and a confirmation measurement has to be done, the second strand of hair would usually be measured. If the second strand was taken from a different head area, different concentrations can be expected based on the presented results. This leads to uncertainty about the validity of the measurements. A possible solution to this problem would be to homogenize all hair strands prior to analysis. An aliquot of the homogenate should then be set aside for reanalysis in any case of doubt. This procedure should lead to reproducible results. However, by homogenizing all the available hair, the possibility of doing further investigations, such as segmental analysis, would be lost. Alternatively two strands immediately next to each other could be sampled, as has recently been suggested by the European Workplace Drug Testing Society.[26] This should limit the concentration differences between the strands, but would negatively impact the haircut of the test subject. One of these approaches must be followed to reliably obtain reproducible results. If this is not done, the laboratory runs the risk of running into the problems described above. The SoHT cut-off values are based on empirical results, which were obtained by measurement of EtG in hair samples of many persons taken at the vertex posterior by different groups.[16,27–29] These cut-off values need not necessarily be suitable for hair samples taken at other head locations. This is not so problematic for the EtG abstinence cut-off at 7 pg/mg, as higher values show that the person was very likely not abstinent. However, for the cut-off for social to excessive drinking at 30 pg/mg this constitutes a large problem. If hair is taken from a spot with a very high EtG incorporation rate compared to the rest of the head, the test subject might be classified as having an excessive alcohol consumption behaviour, which can have great legal consequences. However, no strand of the volunteer showed concentrations above or even close to the 30 pg/mg cut-off. Additionally, it is agreed upon that alcohol consumption is generally underestimated when based on EtG results in hair.[7,30–32] An independent biomarker, such as the fatty acid ethyl esters, could be used as a plausibility control.[28,33] Still, the results should always be viewed in the larger context of the case and a decision should never be based solely on the results of the hair analysis, as is explicitly stated in the SoHT consensus[11] and also was established by the Federal Supreme Court of Switzerland.[34]

The variation of the caffeine concentrations across the head was quite large with a factor of ten. The suitability of caffeine as a model substance for drugs of abuse is unknown. However, the amine group is a common feature of caffeine and most drugs of abuse, meaning they share some similarity. For analysis of cases where a zero drug tolerance policy is in place (like in cases pertaining to re-granting of driving licences) the sampling in places with generally higher incorporation rates might lead to higher sensitivities towards consumption. More investigations are needed to see if caffeine is a good model substance and also if the different drugs of abuse show different intra-head concentration profiles and variabilities.

For monitoring purposes when measuring consumption trends with multiple sampling times, sampling should always be done as close as possible to the previous locations. If sampling is done at different locations, the differing incorporation rates of analyte in these head regions could lead to false interpretations about the consumption behaviour. Therefore, it is proposed that the sampling location should always be recorded and be done at the same location.

The results for the strands which were divided into multiple smaller strands showed low deviations, indicating a relatively good homogeneity within one sampled area. We found decreasing concentrations of EtG and caffeine from the proximal to the distal end of the hair strands. This might point towards a wash-out effect.

A limitation of this study is that it was conducted with only one person and without real drug user hair. The extent of variation and the concentration trends are most likely different in other persons, as the extent and location of sweating is different for every person. It is therefore important to expand the study to further subjects including consumers of illegal drugs, to see if the observed trends in concentration distribution can be verified.

Acknowledgements

We would like to thank the hairdresser of the volunteer, Pia Brandenberg, who spent many hours on meticulously separating and cutting the strands of hair from the volunteer's head. We would also like to thank the laboratory assistant Marco Luginbuehl for helping to prepare the strands for analysis.

References

[1] G. A. A. Cooper, R. Kronstrand, P. Kintz. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci. Int.* **2012**, *218*, 20–24.
[2] F. Pragst, M. A. Balikova. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin. Chim. Acta*. **2006**, *370*, 17–49.
[3] Schweizerische Gesellschaft für Rechtsmedizin, Bestimmung von Ethylglucuronid (EtG) in Haarproben, **2014**. Available at: http://www.sgrm.ch/uploads/media/EtG_FINAL_2014.pdf [19 July 2016].
[4] P. Kintz. *Analytical and Practical Aspects of Drug Testing in Hair*CRC Press, **2007**.
[5] P. Kintz, A. Salomone, M. Vincenti. *Hair Analysis in Clinical and Forensic Toxicology*Academic Press, **2015**.
[6] R. Agius, T. Nadulski, H. G. Kahl, B. Dufaux. Ethyl glucuronide in hair: a highly effective test for the monitoring of alcohol consumption. *Forensic Sci. Int.* **2012**, *218*, 10–14.
[7] C. L. Crunelle, M. Yegles, A. L. van Nuijs, A. Covaci, M. De Doncker, K. E. Maudens, B. Sabbe, G. Dom, W. E. Lambert, P. Michielsen, H. Neels. Hair ethyl glucuronide levels as a marker for alcohol use and abuse: a review of the current state of the art. *Drug Alcohol Depend.* **2014**, *134*, 1–11.
[8] J. C. Kissack, J. Bishop, A. L. Roper. Ethylglucuronide as a biomarker for ethanol detection. *Pharmacotherapy*. **2008**, *28*, 769–781.
[9] H. Dahl, N. Stephanson, O. Beck, A. Helander. Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. *J. Anal. Toxicol.* **2002**, *26*, 201–204.
[10] G. Hoiseth, J. P. Bernard, R. Karinen, L. Johnsen, A. Helander, A. S. Christophersen, J. Morland. A pharmacokinetic study of ethyl glucuronide in blood and urine: applications to forensic toxicology. *Forensic Sci. Int.* **2007**, *172*, 119–124.
[11] Society of Hair Testing. Consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption, **2016**. Available at: http://www.soht.org/index.php/consensus [17 November 2016].
[12] D. Cappelle, H. Neels, M. Yegles, J. Paulus, A. L. van Nuijs, A. Covaci, C. L. Crunelle. Gas chromatographic determination of ethyl glucuronide in hair: comparison between tandem mass spectrometry and single quadrupole mass spectrometry. *Forensic Sci. Int.* **2015**, *249*, 20–24.
[13] A. M. Baumgartner, P. F. Jones, W. A. Baumgartner, C. T. Black. Radioimmunoassay of hair for determining opiate-abuse histories. *J. Nucl. Med.* **1979**, *20*, 748–752.
[14] F. Dussy, N. Carson, S. Hangartner, T. Briellmann. Is one hair lock really representative? *Drug Test. Anal.* **2014**, *6*, 5–8.
[15] J. Schrader, M. Rothe, F. Pragst. Ethyl glucuronide concentrations in beard hair after a single alcohol dose: evidence for incorporation in hair root. *Int. J. Leg. Med.* **2012**, *126*, 791–799.
[16] M. Yegles, A. Labarthe, V. Auwarter, S. Hartwig, H. Vater, R. Wennig, F. Pragst. Comparison of ethyl glucuronide and fatty acid ethyl ester concentrations in hair of alcoholics, social drinkers and teetotallers. *Forensic Sci. Int.* **2004**, *145*, 167–173.
[17] T. M. Binz, M. R. Baumgartner, T. Kraemer. The influence of cleansing shampoos on ethyl glucuronide concentration in hair analyzed with an optimized and validated LC-MS/MS method. *Forensic Sci. Int.* **2014**, *244*, 20–24.
[18] H. Kharbouche, F. Sporkert, S. Troxler, M. Augsburger, P. Mangin, C. Staub. Development and validation of a gas chromatography-negative chemical ionization tandem mass spectrometry method for the determination of ethyl glucuronide in hair and its application to forensic toxicology. *J. Chromatogr. B*. **2009**, *877*, 2337–2343.
[19] F. Pragst, M. Yegles. Determination of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in hair: a promising way for retrospective detection of alcohol abuse during pregnancy? *Ther. Drug Monit.* **2008**, *30*, 255–263.
[20] L. Martins Ferreira, T. Binz, M. Yegles. The influence of ethanol containing cosmetics on ethyl glucuronide concentration in hair. *Forensic Sci. Int.* **2012**, *218*, 123–125.
[21] M. R. Harkey. Anatomy and physiology of hair. *Forensic Sci. Int.* **1993**, *63*, 9–18.
[22] B. M. Appenzeller, M. Schuman, M. Yegles, R. Wennig. Ethyl glucuronide concentration in hair is not influenced by pigmentation. *Alcohol Alcohol.* **2007**, *42*, 326–327.
[23] M. Rothe, F. Pragst, S. Thor, J. Hunger. Effect of pigmentation on the drug deposition in hair of grey-haired subjects. *Forensic Sci. Int.* **1997**, *84*, 53–60.
[24] L. Tsanaclis, R. Kingston, J. Wicks. Testing for alcohol use in hair: is ethyl glucuronide (EtG) stable in hair? *Ann. Toxicol. Anal.* **2009**, *21*, 67–71.
[25] R. Agius, L. M. Ferreira, M. Yegles. Can ethyl glucuronide in hair be determined only in 3 cm hair strands? *Forensic Sci. Int.* **2012**, *218*, 3–9.
[26] A. Salomone, L. Tsanaclis, R. Agius, P. Kintz, M. R. Baumgartner. European guidelines for workplace drug and alcohol testing in hair. *Drug Test. Anal.* **2016**, *8*, 996–1004.
[27] H. Kharbouche, M. Faouzi, N. Sanchez, J. B. Daeppen, M. Augsburger, P. Mangin, C. Staub, F. Sporkert. Diagnostic performance of ethyl glucuronide in hair for the investigation of alcohol drinking behavior: a comparison with traditional biomarkers. *Int. J. Leg. Med.* **2012**, *126*, 243–250.
[28] S. Suesse, F. Pragst, T. Mieczkowski, C. M. Selavka, A. Elian, H. Sachs, M. Hastedt, M. Rothe, J. Campbell. Practical experiences in application of hair fatty acid ethyl esters and ethyl glucuronide for detection of chronic alcohol abuse in forensic cases. *Forensic Sci. Int.* **2012**, *218*, 82–91.
[29] A. Salomone, M. R. Baumgartner, T. Lombardo, E. Alladio, D. Di Corcia, M. Vincenti. Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: comparison of

positivity rates and appraisal of cut-off values. *Forensic Sci. Int.* **2016**, *267*, 60–65.

[30] L. Politi, L. Morini, F. Leone, A. Polettini. Ethyl glucuronide in hair: is it a reliable marker of chronic high levels of alcohol consumption? *Addiction*. **2006**, *101*, 1408–1412.

[31] R. Boscolo-Berto, G. Viel, M. Montisci, C. Terranova, D. Favretto, S. D. Ferrara. Ethyl glucuronide concentration in hair for detecting heavy drinking and/or abstinence: a meta-analysis. *Int. J. Leg. Med.* **2013**, *127*, 611–619.

[32] R. Kronstrand, L. Brinkhagen, F. H. Nystrom. Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months. *Forensic Sci. Int.* **2012**, *215*, 51–55.

[33] M. Hastedt, S. Herre, F. Pragst, M. Rothe, S. Hartwig. Workplace alcohol testing program by combined use of ethyl glucuronide and fatty acid ethyl esters in hair. *Alcohol Alcohol.* **2012**, *47*, 127–132.

[34] Federal Supreme Court of Switzerland, BGE 1C_809/2013, **2014**.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Supporting Information

Table SI. 1

MS parameters used for the determination of EtG

Analyte	Dwell Time [ms]	Declustering Potential (DP) [V]	Entrance Potential (EP) [V]	Collision Energy (CE) [V]	Cell Exit Potential (CXP) [V]
EtG - 85.1 (MS^3)	-	-70	-10	-20	-
EtG - 75.0 (MRM)	30	-70	-10	-21	-16
EtG - 55.0 (MRM)	30	-70	-10	-42	-16
EtG-D5 - 75.0 (MRM)	20	-70	-10	-21	-16
EtG-D5 - 55.0 (MRM)	20	-70	-10	-42	-16

MS^3-specific Parameters							
Ion-Trap Fill Time	Maximum Fill Time [msec]	TIC Target	Scan Rate [Da/sec]	Scan Range [ms/z]	Number of Summed Scans	Excitation Energy (AF2) [V]	Q3 Entry Barrier Potential [V]
"Dynamic"	150	10E7	10000	70-95	2	0.08	8

Table SI. 2

MS parameters used for the determination of caffeine

Analyte	Dwell Time [ms]	Declustering Potential [V]	Entrance Potential [V]	Collision Energy [V]	Cell Exit Potential [V]
Caf - 138.1 (MRM)	100	60	10	27	10
Caf - 110.1 (MRM)	100	60	10	35	10
Caf - 83.0 (MRM)	100	60	10	42	10
Caf-D9 - 144.0 (MRM)	100	60	10	30	10
Caf-D9 - 116.2 (MRM)	100	60	10	35	10
Caf-D9 - 126.0 (MRM)	100	60	10	47	10

Table SI. 3

Comparison of mean results for four international proficiency samples to results obtained with the new LC-MS^3/MRM and a validated GC-MS method.

Proficiency Test #	Mean All Labs [pg/mg]	Validated GC-MS [pg/mg]	LC-MS^3/MRM [pg/mg]	GC-MS Deviation [%]	LC-MS^3/MRM Deviation [%]
1	27.2	26	28.2	-4.4	+3.8
2	23.1	21.6	21.6	-6.5	-6.6
3	13.1	9.4	13.6	-28.2	+3.8
4	8.7	9.2	9.9	+5.7	+13.4

Table SI. 4

EtG and caffeine results for all measured samples are shown. The sample names correspond to the description on the projections.

Sample Name	EtG [pg/mg]	Caffeine [ng/mg]	Sample Name	EtG [pg/mg]	Caffeine [ng/mg]	Sample Name	EtG [pg/mg]	Caffeine [ng/mg]
1.1	11.9	7.5	5.8	14.0	2.4	8.7	12.7	3.2
1.2	13.9	4.9	5.9	16.5	4.9	8.8	12.7	3.5
1.3	18.0	3.7	5.10	16.6	4.8	8.9	12.5	4.3
2.1	13.0	7.2	5.11	10.8	7.7	8.10	10.5	4.7
2.2	14.9	5.6	5.12	10.5	8.8	8.11	10.8	5.3
2.3	16.9	3.4	6.1	15.3	5.4	8.12	6.8	7.7
2.4	18.3	3.1	6.2	13.1	2.9	9.1	10.8	12.0
2.5	16.1	3.0	6.3	13.3	2.1	9.2	15.3	6.4
3.1	16.4	9.0	6.4	13.7	1.4	9.3	14.9	4.8
3.2	14.9	5.4	6.5	15.6	1.1	9.4	11.4	2.7
3.3	16.3	4.0	6.6	14.4	1.6	9.5	15.8	3.7
3.4	16.9	3.6	6.7	16.5	2.6	9.6	13.9	2.8
3.5	16.8	2.8	6.8	15.3	2.7	9.7	12.4	3.4
3.6	18.3	2.8	6.9	14.3	4.6	9.8	13.3	4.6
3.7	16.0	3.1	6.10	12.7	5.5	9.9	9.9	4.7
3.8	16.1	4.0	6.11	11.2	6.7	9.10	10.5	6.0
3.9	16.5	5.9	6.12	8.5	7.8	9.11	9.2	8.2
3.10	16.7	6.9	7.1	14.9	4.9	10.1	14.3	8.0
4.1	15.6	7.3	7.2	9.9	3.1	10.2	14.0	5.1
4.2	14.5	3.6	7.3	9.8	1.3	10.3	13.7	3.7
4.3	14.4	2.7	7.4	11.9	1.4	10.4	16.8	3.3
4.4	18.8	3.1	7.5	14.1	1.7	10.5	12.8	3.9
4.5	20.2	2.8	7.6	11.0	1.3	10.6	12.1	4.0
4.6	17.3	2.6	7.7	11.1	1.9	10.7	12.4	5.3
4.7	18.1	3.4	7.8	13.3	3.3	10.8	12.9	6.8
4.8	16.8	4.2	7.9	12.2	5.1	10.9	10.3	9.4
4.9	13.4	5.4	7.10	9.9	4.8	11.1	12.7	9.5
4.10	15.1	6.9	7.11	8.6	5.1	11.2	13.2	6.9
5.1	17.8	7.4	7.12	7.4	7.4	11.3	15.9	5.8
5.2	16.4	5.2	8.1	10.4	9.5	11.4	11.3	4.4
5.3	15.6	2.3	8.2	12.9	4.7	11.5	11.4	4.7
5.4	13.6	1.7	8.3	12.1	3.6	11.6	14.9	7.5
5.5	16.1	1.5	8.4	11.1	1.9	12.1	12.0	9.0
5.6	12.2	1.9	8.5	14.8	1.8	12.2	10.6	7.1
5.7	15.3	2.4	8.6	13.7	2.4			

Table SI. 5

EtG [pg/mg] and caffeine [ng/mg] results of the intra-sample area strand homogeneity experiment. The expanded measurement uncertainty of the method is given in parenthesis for the mean of the results.

Sample Area	8.8	7.8	6.8	3.3	6.5	8.11
			EtG [pg/mg]			
1	12.1	12.6	15.4	17.3	16.3	9.7
2	11.5	13.6	14.0	17.0	15.4	12.0
3	14.5	13.3	16.0	15.0	14.8	10.8
4	12.8	13.8	15.7	15.7	16.1	
Mean	12.7 (±2.0)	13.3 (±2.1)	15.3 (±2.4)	16.3 (±2.6)	15.6 (±2.5)	10.8 (±1.7)
			Caffeine [ng/mg]			
1	3.6	3.8	3.2	4.1	1.2	5.9
2	3.4	3.4	2.4	3.6	1.1	4.9
3	3.5	2.9	2.2	3.8	1.0	5.0
4	3.7	3.0	2.9	4.3	1.1	
Mean	3.5 (±0.5)	3.3 (±0.5)	2.7(±0.4)	4.0(±0.5)	1.1(±0.2)	5.3(±0.7)

Table SI. 6

EtG [pg/mg] and caffeine [ng/mg] results of the strand segmentation experiment. The ratio of the proximal to distal segment is given.

Sample Area	6.7	3.4	10.4	6.6	9.2	6.4	6.11
EtG [pg/mg]							
0-1 cm	23.9	22.3	22.5	21.9	22.0	21.1	16.2
1-2 cm	15.2	14.7	15.2	16.7	13.9	14.6	10.1
2-3 cm	11.8	14.8	12.6	11.0	8.6	7.9	8.7
Ratio 0-1 cm/2-3 cm	2.0	1.5	1.8	2.0	2.6	2.7	1.9
Caffeine [ng/mg]							
0-1 cm	3.7	4.0	4.5	2.5	6.1	1.5	5.4
1-2 cm	1.8	2.9	2.9	1.8	4.7	1.0	4.5
2-3 cm	1.1	2.3	2.0	1.4	3.9	0.8	5.1
Ratio 0-1 cm/2-3 cm	3.2	1.7	2.2	1.7	1.6	1.9	1.1

3.2. Project 2: Sample Preparation Method for the Combined Extraction of Ethyl Glucuronide and Drugs of Abuse in Hair

About Project 2

This publication describes a combined hair sample preparation method for EtG and DoA for subsequent analysis using LC-MS^3/MS^2. This method allows substantially reducing the workload when analyzing samples for both these parameter groups while reducing the amount of required hair.

Authors: Ulf Meier, Thomas Briellmann, Eva Scheurer, Franz Dussy

Contributions of Ulf Meier:

- Development of the combined sample preparation method
- Conducting the sample preparation, measurements and data evaluation
- Writing the article

CRediT Statement:

Ulf Meier:	Conceptualization, formal analysis, investigation, methodology, project administration, writing – original draft
Thomas Briellmann:	Writing – review & editing
Eva Scheurer:	Supervision, writing – review & editing
Franz Dussy:	Conceptualization, supervision, writing – review & editing

Status: Published

Received: 29 March 2017 | Revised: 19 September 2017 | Accepted: 19 September 2017

DOI: 10.1002/dta.2314

WILEY

RESEARCH ARTICLE

Sample preparation method for the combined extraction of ethyl glucuronide and drugs of abuse in hair

Ulf Meier | Thomas Briellmann | Eva Scheurer | Franz Dussy

Forensic Chemistry and Toxicology, Institute of Forensic Medicine of the University of Basel, Basel, Switzerland

Correspondence
Ulf Christian Juhl Meier, Institute of Forensic Medicine of the University of Basel, Forensic Chemistry and Toxicology, Basel, CH 4056, Switzerland.
Email: ulf.meier@bs.ch; ulfmeier.um@gmail.com

Abstract

Often in hair analysis, a small hair sample is available while the analysis of a multitude of structurally diverse substances with different concentration ranges is demanded. The analysis of the different substances often requires different sample preparation methods, increasing the amount of required hair sample. When segmental hair analysis is necessary, the amount of hair sample needed is further increased. Therefore, the required sample amount for a full analysis can quickly exceed what is available. To combat this problem, a method for the combined hair sample preparation using a single extraction procedure for analysis of ethyl glucuronide with liquid chromatography-multistage fragmentation mass spectrometry/multiple reaction monitoring (LC–MS^3/MRM) and common drugs of abuse with LC–MRM was developed. The combined sample preparation is achieved by separating ethyl glucuronide from the drugs of abuse into separate extracts by fractionation in the solid-phase extraction step during sample clean-up. A full validation for all substances for the parameters selectivity, linearity, limit of detection, limit of quantification, accuracy, precision, matrix effects, and recovery was successfully completed. The following drugs of abuse were included in the method: Amphetamine; methamphetamine; 3,4-methylenedioxy-N-methylamphetamine (MDMA); 3,4-methylenedioxyamphetamine (MDA); 3,4-methylenedioxy-N-ethylamphetamine (MDE); morphine; 6-monoacetylmorphine; codeine; acetylcodeine; cocaine; benzoylecgonine; norcocaine; cocaethylene; methadone; 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methylphenidate. In conclusion, as only 1 sample preparation is needed with 1 aliquot of hair, the presented sample preparation allows an optimal analysis of both ethyl glucuronide and of the drugs of abuse, even when the sample amount is a limiting factor.

KEYWORDS

hair analysis, ethyl glucuronide, drugs of abuse, combined extraction, sample preparation

1 | INTRODUCTION

Many drugs and drug metabolites are incorporated into hair after consumption.[1] As they are trapped within the hair and show long-term stability, hair allows a long retrospective window of detection, making it useful for forensic questions like abstinence controls,[2,3] driving license-regranting procedures, workplace drug testing, and child custody cases.[1] Ethanol and the drugs of abuse (DOA) are 2 substance groups of interest in these cases.[1] The biomarker of choice for determining ethanol consumption in hair is ethyl glucuronide (EtG), which has been shown in many studies to be a suitable biomarker for differentiating between abstinence, social drinking, and chronic excessive drinking.[4-6] For the DOA, the substances themselves and various metabolites are measured.[2,3,7] In addition to the long detection window, head hair has the advantage of allowing a temporal resolution of consumption based on the segment of the hair that is analyzed, where 1 cm of head hair corresponds roughly to a 1-month time period.[1,5,8-11]

Hair analysis is usually done using ca. 10–50 mg of hair.[12-15] The hair is cut and/or ground and subjected to extraction procedures after which the extract is enriched and clean-up steps are performed. Often, hair samples need to be analyzed for both EtG and DOA, typically using different extraction procedures for the different substance groups. Therefore, multiple aliquots of hair and multiple sample preparations are required. If segmental hair analysis is required in such a case, a large amount of hair is needed, which can quickly exceed the

readily available amount. To alleviate this problem, a sample preparation procedure was developed and validated for the simultaneous extraction and preparation of hair samples for EtG and for common DOA for subsequent measurement with liquid chromatography-multistage fragmentation mass spectrometry/multiple reaction monitoring (LC–MS^3/MRM) for EtG and LC–MRM for the DOA. This is achieved by separating EtG and the DOA into different extracts using the different retention characteristics of the substances on an anion exchange mixed mode solid-phase extraction (SPE) cartridge. In addition to reducing the amount of required hair, the simultaneous sample preparation of both EtG and DOA can slightly reduce the cost and the required time for hair analysis in cases where analysis of EtG as well as DOA is required.

2 | MATERIALS AND METHODS

2.1 | Chemicals and instrumentation

All used reference and internal standards were supplied by Lipomed (Arlesheim, Switzerland). For EtG (EtG-D5) and for all DOA except Norcocaine (calculated with cocaine-D3), a deuterated internal standard was used. The used DOA deuterated internal standards are listed in Table 1. An EtG stock solution at a concentration of 10 ng/µL in methanol was prepared. A stock mix containing all DOA standards at a concentration of 10 ng/µL in acetonitrile was prepared. Working standard solutions with various concentrations were prepared from the stock solutions as needed. An internal standard working mix containing all DOA internal standards at 5 ng/µL in acetonitrile was used. For EtG-D5, a working solution containing 100 pg/µL in methanol was used. Standards and internal standards were stored at 4°C. The LC–MS solvents water, acetonitrile and methanol were all obtained in analytical grade purity from Machery-Nagel AG (Oensingen, Switzerland). Formic acid puriss p.a. (98%), ammonium formate (≥ 99.0%) and ammonium hydroxide solution (25%) were obtained from Sigma-Aldrich (Buchs, Switzerland). 2-propanol (≥ 99.5%) and acetone (≥ 99.5%) used for washing the hair were purchased from Roth (Arlesheim, Switzerland). Deionized water used for washing the hair was produced in house. An Oasis Max 60 mg, 3 mL cartridge purchased from Waters (Daettwil, Switzerland) was used for the SPE.

An MM 200 ball mill (Retsch, Haan, Germany) was used for hair pulverization. All analysis were carried out with an Ultimate 3000 high performance liquid chromatograph (Thermo Fisher Scientific, Reinach, Switzerland) coupled to a 5500 QTrap triple quadrupole mass spectrometer (Sciex, Brugg, Switzerland).

2.2 | Sample preparation

Hair strands weighing around 50–100 mg were washed with 3 mL water, followed by 3 mL 2–propanol and finally 3 mL acetone. The strands were dried under a soft stream of nitrogen or by leaving them over night. The hair strands were subsequently cut into snippets of around 1 mm by scissors. Approximately 20 mg of the hair snippets were pulverized in a 2 mL Eppendorf tube equipped with two 5 mm stainless steel balls with a ball mill operated at a frequency of 30 Hz for 7 minutes. To the powdered hair 5 µL EtG-D5 solution (500 pg) and 4 µL DOA internal standard mix (20 ng each) were added. The hair was extracted for 4 hours with 1.5 mL water:methanol 1:1 using an overhead shaker. The sample was centrifuged at 13 000 rpm for 10 minutes. EtG was separated from the DOA by using an SPE. For this, the supernatant was added to an Oasis Max SPE cartridge, which had been conditioned with 2 mL methanol followed by 2 mL water. The DOA are only partially retained on the SPE column. Therefore, the fraction of the supernatant passing through the SPE cartridge was collected. The cartridge was washed with 2 mL MeOH and the wash solution containing the remaining DOA was combined with the first fraction and set aside. The column was then washed using first 1 mL of water followed by 2 mL of MeOH. These 2 washing fractions were discarded. The cartridge was dried for 10 minutes under vacuum. EtG was eluted using 2 mL methanol with 2% formic acid. Both the DOA and EtG containing extracts were dried separately under a gentle nitrogen stream. The DOA extract was reconstituted in 0.5 mL ammonium formate buffer (200 nM, pH 5), while the EtG extract was reconstituted in 50 µL water.

2.3 | LC–MS methods

LC–MS methods are routinely used in the authors' lab, are validated and are in the accredited part of the laboratory.

2.3.1 | EtG

The LC–MS^3/MRM method for the measurement of EtG has been described elsewhere.[16] Briefly, a Hypercarb 100 x 2.1 mm 3 µm column (Thermo Fisher Scientific, Reinach, Switzerland), protected by a Phenomenex KrudKatcher Ultra 0.5 µm 316 stainless-steel depth filter pre-column (Brechbühler, Schlieren, Switzerland), with a flow rate of 400 µL/min was used, with the following LC program: 1% fresh ammonia solution was used for the gradient program with 100% (1-minute hold) to 90% at 4 minutes, 10 % at 8 minutes (2-minute hold), 0% at 10.2 minutes and 100% at 12 minutes (hold 3 minutes) adjusted with acetonitrile to 100%. EtG was measured with injection of 20 µL of sample. A method with both MRM and MS^3 (done with the linear ion trap) experiments was used. The instrument was operated in negative electrospray ionization (ESI) mode using an ion-spray voltage of -4500 kV, a source temperature (TEM) of 600°C, a curtain gas (CUR) of 40 psi nitrogen and nebulizer (GS1) and heating (GS2) gas of 65 and 60 psi, respectively. The collision associated dissociation gas (CAD) was set to 11. Further instrument parameters and used mass transitions are described in Tables 2 and 3. A total cycle time of 0.41 seconds resulted for the method. The total runtime of the method was 16 minutes with EtG eluting at 3.9 minutes.

2.3.2 | DOA

The following DOA were quantified: Amphetamine; methamphetamine; 3,4-methylenedioxy-N-methylamphetamine (MDMA); 3,4-methylenedioxyamphetamine (MDA); 3,4-methylenedioxy-N-ethylamphetamine (MDE); morphine; 6-monoacetylmorphine (MAM); codeine; acetylcodeine (AcCo); cocaine; benzoylecgonine (BE); norcocaine (NorCoc); cocaethylene (CE); methadone (MTD);

TABLE 1 Expected retention times, MRM transitions, and MS parameters used for the DOA method. Substances are ordered according to their retention time

Drugs of abuse	Q1 [Da]	Q3 [Da]	RT [Min]	DP [V]	EP [V]	CE [V]	CXP [V]
Morphine 1	286.1	165.1	2.1	85	10	55	12
Morphine 2	286.1	152.1	2.1	85	10	80	12
Morphine 3	286.1	128.1	2.1	85	10	78	12
Amphetamine 1	136.1	119.1	3.6	13	6	12	11
Amphetamine 2	136.1	91	3.6	13	6	22	11
Codeine 1	300.2	152.1	3.85	70	10	85	12
Codeine 2	300.2	165.1	3.85	70	10	51	12
Methamphetamine 1	150.1	119.1	3.9	45	6	15	13
Methamphetamine 2	150.1	91	3.9	45	6	23	13
MDA 1	180.2	135.1	4.1	44	10	25	9
MDA 2	180.2	105.1	4.1	44	10	29	9
MDA 3	180.2	77.1	4.1	44	10	51	9
MDMA 1	194.2	163.2	4.3	50	10	19	12
MDMA 2	194.2	105.2	4.3	50	10	37	12
MAM 1	328.2	165.1	4.45	100	10	49	12
MAM 2	328.2	211.1	4.45	100	10	35	12
MDE 1	208.3	163.2	4.7	50	10	21	12
MDE 2	208.3	105.2	4.7	50	10	40	12
BE 1	290.1	168.2	5.52	65	10	27	12
BE 2	290.1	77.1	5.52	65	10	82	12
Methylphenidate 1	234.2	84.1	6.05	60	10	29	13
Methylphenidate 2	234.2	56.1	6.05	60	10	66	13
Cocaine 1	304.2	182.2	6.1	80	10	30	12
Cocaine 2	304.2	82.1	6.1	80	10	42	12
AcCo 1	342.2	225	6.1	100	10	37	12
AcCo 2	342.2	165.1	6.1	100	10	58	12
NorCoc 1	290.2	168.201	6.6	36	10	22	11
NorCoc 2	290.2	136.1	6.6	36	10	29	11
NorCoc 3	290.2	77.101	6.6	36	10	79	11
CE 1	318.3	196.3	7.2	75	10	30	12
CE 2	318.3	82.2	7.2	75	10	55	12
EDDP 1	278.2	249.1	8.9	90	10	32	11
EDDP 2	278.2	234.1	8.9	90	10	40	11
EDDP 3	278.2	219.1	8.9	90	10	53	11
MTD 1	310.4	265.3	10.9	65	10	22	12
MTD 2	310.4	105.2	10.9	65	10	40	12
IS Morphine-D3 1	289.1	165.1	2.1	85	10	55	12
IS Morphine-D3 2	289.1	152.1	2.1	85	10	80	12
IS Morphine-D3 3	289.1	128.1	2.1	85	10	78	12
IS Amphetamine-D6 1	142.1	125.1	3.6	13	6	12	11
IS Amphetamine-D6 2	142.1	93	3.6	13	6	22	11
IS Codeine-D3 1	303.2	152.1	3.85	70	10	85	12
IS Codeine-D3 2	303.2	165.1	3.85	70	10	51	12
IS Methamphetamine-D5 1	155.1	121.1	3.9	45	6	15	13
IS Methamphetamine-D5 2	155.1	92	3.9	45	6	23	13
IS MDA-D5 1	185.2	138.1	4.1	44	10	25	9
IS MDA-D5 2	185.2	110.1	4.1	44	10	29	9
IS MDA-D5 3	185.2	80.1	4.1	44	10	51	9
IS MDMA-D5 1	199.2	165	4.3	50	10	19	12
IS MDMA-D5 2	199.2	107.2	4.3	50	10	37	12

(Continues)

TABLE 1 (Continued)

Drugs of abuse	Q1 [Da]	Q3 [Da]	RT [Min]	DP [V]	EP [V]	CE [V]	CXP [V]
IS MAM-D3 1	331.1	165.1	4.45	100	10	49	12
IS MAM-D3 2	331.1	211.1	4.45	100	10	35	12
IS MDE-D5 1	213.3	163.2	4.7	50	10	21	12
IS MDE-D5 2	213.3	105.2	4.7	50	10	40	12
IS BE-D3 1	293.1	171.2	5.5	65	10	27	12
Is BE-D3 2	293.1	77.1	5.5	65	10	82	12
IS Methylphenidate -D10 1	244.05	93.1	6.05	60	10	29	13
IS Methylphenidate -D10 2	244.05	61	6.05	60	10	66	13
IS Cocaine-D3 1	307.2	185.2	6.1	80	10	30	12
IS cocaine-D3 2	307.2	85.1	6.1	80	10	42	12
IS AcCo-D3 1	345.1	225	6.1	100	10	37	12
IS AcCo-D3 2	345.1	165.1	6.1	100	10	58	12
IS CE-D3 1	321.1	199.1	7.2	75	10	30	12
IS CE-D3 2	321.1	85.1	7.2	75	10	55	12
IS EDDP-D3 1	281.1	249.1	8.9	90	10	32	11
IS EDDP-D3 2	281.1	234.1	8.9	90	10	40	11
IS EDDP-D3 3	281.1	219.1	8.9	90	10	53	11
IS MTD-D3 1	319.4	268.2	10.9	65	10	22	12
IS MTD-D3 2	319.4	105	10.9	65	10	40	12

(Q1/Q3) Mass filtered in the 1st/3rd Quadrupole; (RT) Expected Retention Time used for scheduled MRM; (DP) Declustering Potential; (EP) Entrance Potential; (CE) Collision Energy; (CXP) Cell Exit Potential

TABLE 2 Retention time, MS^3 transition, and MS parameters used for the EtG method

EtG-113-85	Precursor Ion [Da]	Product Ion [Da]	2nd Generation Ion [DA]	RT [min]	DP [V]	EP [V]	CE [V]
	221.1	113.0	85.1	3.90	-70	-10	-20
	Max. Fill Time [msec]	**TIC Target [Counts]**	**Scan Rate [Da/sec]**	**Scan Range [ms/z]**	**Summed Scans**	**Excitation Energy [V]**	**Q3 Entry Barrier Potential [V]**
	150	10E7	10000	70-95	2	0.08	8

(RT) Retention Time; (DP) Declustering Potential; (EP) Entrance Potential; (CE) Collision Energy

TABLE 3 Retention time, MRM transitions, and MS parameters used for the EtG method

	Q1 [Da]	Q3 [Da]	RT [min]	Dwell Time [msec]	DP [V]	EP [V]	CE [V]	CXP [V]
EtG-55	221.1	55.0	3.90	30	-70	-10	-42	-16
EtG-75	221.1	75.0	3.90	30	-70	-10	-21	-16
EtG75-D5	226.0	75.0	3.90	20	-70	-10	-21	-16
EtG55-D5	226.0	55.0	3.90	20	-70	-10	-42	-16

(Q1/Q3) Mass filtered in the 1st/3rd Quadrupole; (RT) Retention Time; (DP) Declustering Potential; (EP) Entrance Potential; (CE) Collision Energy; (CXP) Cell Exit Potential

2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and methylphenidate.

The LC–MRM method for the measurement of DOA was adapted from Miller.[17] The analysis of DOA was done using a Phenomenex Kinetex 2.6 µm XB-C18 100 Å 50 x 2.1 mm column (Brechbühler, Schlieren, Switzerland), protected by a Phenomenex KrudKatcher Ultra 0.5 µm 316 stainless-steel depth filter pre-column (Brechbühler, Schlieren, Switzerland), and a flow rate of 500 µL/min with the following LC program: ammonium formate buffer (10 mM ammonium formate set to pH 3.4 with formic acid) was used for the gradient program with 99.5 % (1.5 min hold) to 90% at 2 minutes, 40% at 12 minutes, and 20% at 13 minutes (2 minute hold; 15 minutes total time) adjusted to 100% with methanol. DOA were measured with injection of 5 µL of sample in positive atmospheric pressure chemical ionization (APCI) mode. The instrument was operated with a TEM of 450°C, CUR gas of 30 psi and a GS1 gas of 50 psi with a nebulizer current (NC) of 3 V. The CAD gas was set to 8. Substance specific instrument parameters and mass transitions are described in Table 1. Data acquisition was done using a scheduled MRM program with a target cycle time of 0.3 seconds.

2.4 | Validation

Validation of the method was performed according to the guidelines of the German-speaking society of toxicology and forensic chemistry GTFCh (Gesellschaft für Toxikologie und Forensische Chemie).[18] Unless specified otherwise, all validation parameters were evaluated with a pool of blank hair (3 children) and standards were spiked to the powdered hair. Selectivity was tested in 6 blank hair extracts of children (3) and alcohol-abstaining adults (3) without internal standard as well as in 2 blank child hair extracts spiked with internal standard. Linearity was tested with 6 calibration points for EtG (5, 20, 40, 60, 80, 100 pg/mg) and 7 for DOA (50, 500, 2000, 4000, 6000, 8000, 10000 pg/mg) corresponding to the range of interest for hair analysis. Each level was tested in 6 replicates. The calibration curve was estimated using least squared analysis and the squared correlation coefficient (R^2) used to estimate linearity. Mandel's test was used to verify the appropriateness of the linear model (99% significance level). A weighing factor was chosen to minimize the sum of the residuals. Homoscedasticity was checked by using an F-test on the lowest and highest calibration levels (95% significance level). The limit of detection (LOD) and limit of quantification (LOQ) were estimated using EtG/DOA spiked blank hair samples in triplicates and each sample requiring a signal-to-noise ratio of at least 3:1 and 10:1, respectively, and requiring deviation to the expected value below ±20% for the LOQ. Noise was evaluated from ±0.05 minutes before onset/after end of the peak. The lowest tested concentrations for LOD/LOQ were 1 pg/mg for EtG and 5 pg/mg for the DOA.

Accuracy and precision were determined by measuring blank hair samples spiked with low, mid, and high concentrations in duplicate on 8 different days. The extraction efficiency and trueness of the results were additionally evaluated by measuring 7 proficiency test samples for EtG and 2 for the DOA and comparing the results with the median of all participating labs. The proficiency test samples were authentic hair samples obtained from the Society of Hair Testing (SoHT) or from the Swiss inter-laboratory comparison.

Matrix effect and recovery were evaluated as follows: Five different blank hair samples were prepared, with standards spiked to the evaporated SPE extracts (A). The spiked blank hair extracts (A) were compared to solutions of the same concentrations made in water (B) and the matrix effect calculated as A/B*100. The recovery was determined by measurement of 5 spiked blank hair samples (C) from the same sources with the same concentrations and comparing the areas with those obtained from the spiked extracts (recovery = C/A*100).

Unless specified otherwise, all transitions were used as quantifiers and the mean was used for evaluation with each transition having to fulfill the acceptance criteria. Data analysis for all analytes was carried out using the software MultiQuant (version 3.0.2, Sciex, Brugg, Switzerland). All calculations and statistics were done using Microsoft Excel 2010 (Redmond, WA, USA).

3 | RESULTS AND DISCUSSION

The validation for EtG met all the criteria for successful validation set by the GTFCh. The concentration range from 5 pg/mg to 100 pg/mg showed a linear calibration curve with R^2-values of at least 0.999 using a weighing factor of 1/x. The use of the linear model was confirmed by Mandel's test. Homoscedasticity was not given over the entire range. However, the correctness of the calibration was confirmed by measurement of proficiency test samples and comparison with the median of all participating laboratories, as shown in Table 4. The method gave similar results to the median of all participating laboratories with a maximum and average deviation of 10.6% and 3.0%, respectively. There were no interfering signals with any tested blank hair sample with or without internal standard for the MS^3 (221.1 ➔ 113.0 ➔85.0) or MRM (221.1 ➔55.0) transitions. The MRM transition (221.1 ➔ 75.1) showed some interfering signals in the low concentration range, and therefore was only used as a qualifier. Consequently, this transition was not used for the calculation of any validation parameter. Results for LOD, LOQ, accuracy and precision, matrix effect and recovery are shown in Table 5. The LOD (1 pg/mg) and LOQ (3 pg/mg) are similar to other published methods[13,19] and provide adequate sensitivity for interpretation using the SoHT cut-off values of 7 pg/mg for abstinence and 30 pg/mg for social drinking. A chromatogram of a blank hair sample spiked with 5 pg/mg is shown in Figure 1.

The validation for DOA met all the criteria for successful validation set by the GTFCh. The concentration range from 50 pg/mg to 10000 pg/mg showed a linear calibration curve with R^2- values of at least 0.998 using a weighing factor of 1/x. The linear model was confirmed by Mandel's test for all substances. Homoscedasticity was not given for any substance over the entire range. However, the correctness of the calibration was confirmed by comparison with proficiency test samples, as shown in Table 6. No interferences were seen for any DOA in the blank samples with or without internal standard. Results for LOD, LOQ, accuracy and precision, matrix effects, and recoveries are shown in Table 5. The LOQs of the drug metabolites NorCoc, CE and EDDP were 5 pg/mg, which is well below the SoHT recommendation for the cut-off of metabolites of 50 pg/mg.[9] The highest LOQ was 30 pg/mg for MDA, which is well below the cut-off of 200 pg/mg recommended by the SoHT for the DOA.[9,10] The obtained results for the proficiency test samples are generally higher than the median of the participating laboratories. However, in the presented method, samples were pulverized prior to extraction. Many laboratories only cut the hair samples into snippets prior to extraction. It

TABLE 4 Results of the EtG proficiency test samples measured with the combined method and compared to the median of all participating (Samples 1–4: 6 participants, inter-laboratory comparison, Switzerland, 2014; samples 5–7: 30 participants, SoHT, 2016) laboratories

EtG	1	2	3	4	5	6	7
Combined method [pg/mg]	143	7.0	16.2	<LOD	29.3	14.5	22.0
Median of all participation laboratories [pg/mg]	142	7.1	16.4	-	32.8	15.2	22.0
Deviation of combined method to the median of all labs [%]	0.0	-1.4	-1.2	-	-10.6	-4.6	0.0
Mean deviation [%]	-3.0						

TABLE 5 Validation data for EtG and the DOA. Concentration levels: EtG (low: 5 pg/mg; mid: 20 pg/mg; high: 40 pg/mg); DOA (low: 50 pg/mg; mid: 2500 pg/mg; high: 5000 pg/mg)

Substance	LOD [pg/mg]	LOQ [pg/mg]	Accuracy Bias [%]			Intra-day Precision [CV [%]]			Inter-day Precision [CV [%]]			Matrix Effect [% ± RSD]		Recovery [% ± RSD]	
			low	mid	high	low	mid	high	low	mid	high	low	high	low	high
EtG															
EtG	1	3	6.33	2.61	3.98	1.56	4.97	3.74	2.86	4.97	5.69	93.4 ± 9.6	78.1 ± 12.7	78.8 ± 13.5	85.4 ± 21.7
DOA															
Amphetamine	5	5	-11.03	-1.41	-2.64	4.64	3.18	2.24	5.48	3.18	2.24	119.0 ± 12.9	103.5 ± 1.8	80.1 ± 8.1	81.7 ± 3.7
Methamphetamine	5	20	-9.69	-3.71	-1.89	4.46	3.29	1.16	5.68	3.29	1.16	118.2 ± 10.1	102.8 ± 2.3	76.6 ± 9.1	84.7 ± 3.9
MDA	20	30	-8.93	-2.51	-1.44	5.98	2.42	2.17	5.57	2.42	2.17	104.3 ± 15.1	99.5 ± 3.6	80.7 ± 8.5	82.8 ± 3.8
MDE	10	10	-4.65	-2.99	-2.74	4.67	3.59	2.80	6.42	3.59	2.80	101.9 ± 6.1	97.7 ± 2.1	82.2 ± 8.5	87.1 ± 4.0
MDMA	5	20	-4.93	-0.95	-0.70	3.45	4.96	3.20	6.82	4.96	3.20	104.1 ± 7.2	100.1 ± 3.4	80.9 ± 8.8	83.8 ± 5.2
Morphine	10	10	-5.75	-0.47	-0.42	3.93	3.45	1.88	5.89	3.45	1.88	104.5 ± 7.9	104.4 ± 4.0	83.3 ± 8.6	82.0 ± 1.8
MAM	10	10	-6.85	-1.24	-3.28	6.38	2.96	3.50	7.62	2.96	3.50	103.7 ± 8.1	99.6 ± 3.0	83.0 ± 8.1	85.0 ± 2.8
Codeine	5	10	-3.05	2.23	2.55	6.49	4.66	4.70	5.59	4.66	4.70	99.7 ± 17.5	96.3 ± 4.0	89.5 ± 12.6	88.2 ± 3.9
AcCo	5	5	-6.91	-1.51	-1.09	2.47	2.83	1.83	5.98	2.83	1.83	99.9 ± 8.1	98.4 ± 3.0	82.1 ± 7.2	86.0 ± 3.7
Cocaine	10	20	-0.81	-3.33	-1.68	3.48	2.69	2.85	5.78	2.69	2.85	100.9 ± 10.0	98.9 ± 4.0	82.4 ± 10.5	87.3 ± 6.0
BE	5	10	-5.81	-2.78	-2.36	3.83	3.77	1.60	4.41	3.77	1.60	125.0 ± 15.6	124.8 ± 13.6	87.0 ± 12.2	94.3 ± 10.3
CE	5	5	-5.19	-2.10	-1.88	3.03	3.06	1.92	6.71	3.06	1.92	94.6 ± 2.6	98.5 ± 2.6	84.6 ± 7.9	86.5 ± 5.0
NorCoc	5	5	-7.45	-2.74	-2.37	2.73	1.41	4.29	7.20	1.41	4.29	98.7 ± 6.0	99.6 ± 1.8	80.1 ± 5.0	84.1 ± 3.4
MTD	10	20	-0.25	-1.52	-1.35	2.54	2.30	1.87	7.14	2.30	1.87	106.5 ± 4.7	100.7 ± 1.9	81.5 ± 6.3	86.4 ± 4.0
EDDP	5	5	-6.03	-2.56	-2.14	2.70	1.99	1.94	4.81	1.99	1.94	102.5 ± 8.1	98.5 ± 2.3	83.4 ± 10.6	87.0 ± 3.1
Methylphenidate	10	10	-8.10	-3.57	-2.62	4.14	3.23	2.98	6.15	3.23	2.98	108.0 ± 6.8	100.0 ±3.3	78.5 ± 9.2	84.8 ± 5.4

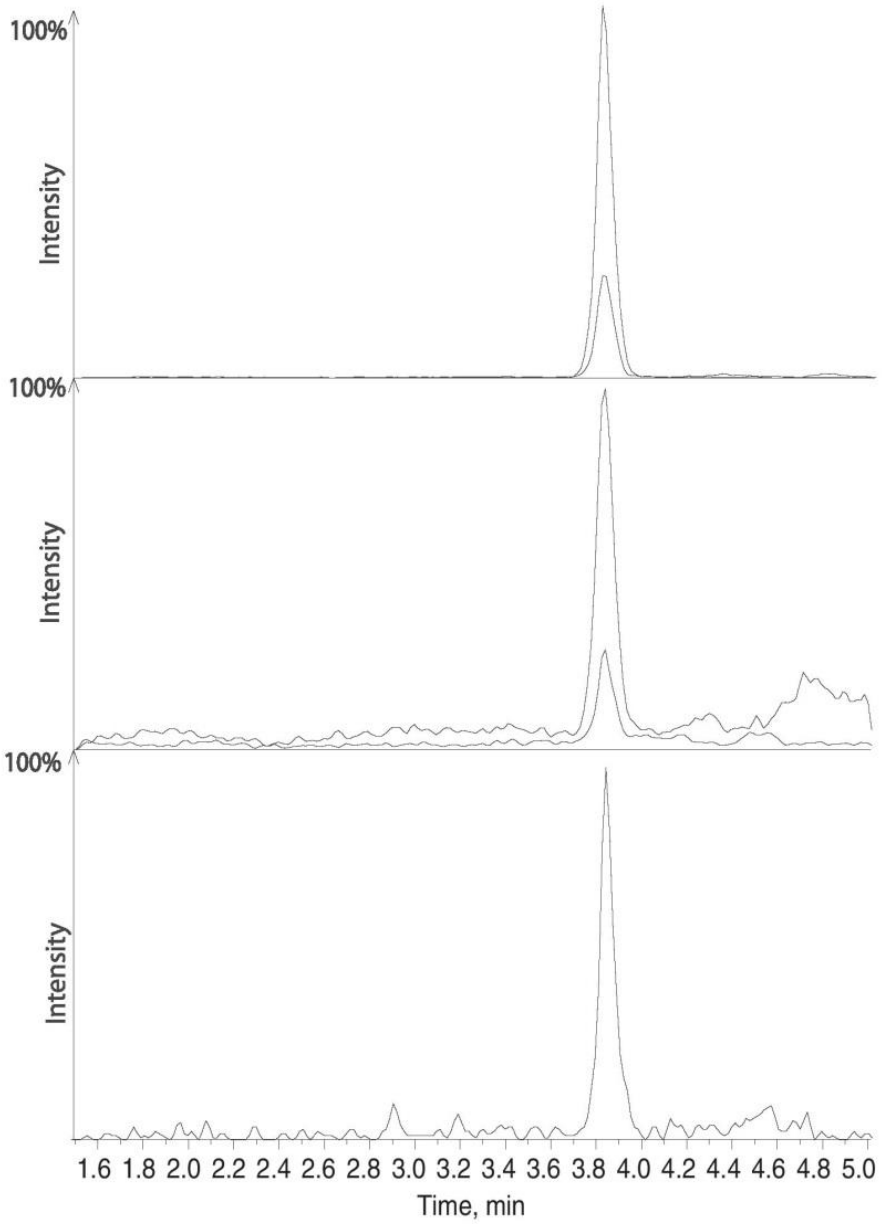


FIGURE 1 Chromatogram of a blank hair sample spiked with 5 pg/mg EtG and 25 pg/mg EtG-D5. (Top) EtG-D5 MRM transitions 226 ➔ 75 and 226 ➔ 55. (Middle) EtG MRM transitions 221 ➔ 75 and 221 ➔ 55. (Bottom) EtG MS^3 transition 221 ➔ 113 ➔ 85. Chromatogram was smoothed with a 3 point smoothing algorithm in the Analyst software.

TABLE 6 Results of the DOA proficiency test samples (SoHT, 2015) measured with the combined method and compared to the median of all participating (48) laboratories

	Sample 1			Sample 2		
DOA	Combined method [pg/mg]	Median [pg/mg]	Deviation to median [%]	Combined method [pg/mg]	Median [pg/mg]	Deviation to median [%]
Amphetamine	170	160	4.0	-	-	-
Methamphetamine	110	130	-12.3	-	-	-
MDMA	150	110	32.9	-	-	-
Morphine	930	950	-1.7	3650	3380	8.2
MAM	3780	3280	15.4	13690	13200	3.8
Codeine	260	220	16.4	1010	790	27.4
Cocaine	6840	5530	23.7	7410	6300	17.6
BE	12140	7450	63.0	11930	8040	48.4

is very likely that pulverization of hair leads to higher extraction efficiencies than only cutting the hair, as has been shown for some DOA and for EtG.[3,20,21] The SoHT recommends pulverization of the hair for EtG[22] and therefore this procedure was followed in the presented method. A chromatogram of a hair sample spiked with 50 pg/mg DOA is shown in Figure 2.

The main advantage of the combined extraction over traditional 1 analyte group extractions is the reduced amount of required hair sample. Especially in segmental analysis, the amount of hair available for each segment can be a limiting factor. The presented method can help alleviate this problem. The combined method involves more work compared to the routine methods employed at the authors' lab if an analysis of only EtG or only DOA is required. However, if both analyte groups are demanded the sample preparation time can be slightly reduced. The routine method used at the authors' lab involves 2 separate sample preparations for DOA and EtG. Briefly, for EtG the hair is pulverized and extracted with 1.5 mL water for at least 1 hour. The extract is centrifuged and the supernatant cleaned using an Oasis Max SPE. The extract is evaporated and finally reconstituted in 50 µL of water. For the DOA sample preparations from Cordero,[23] Romolo,[24]

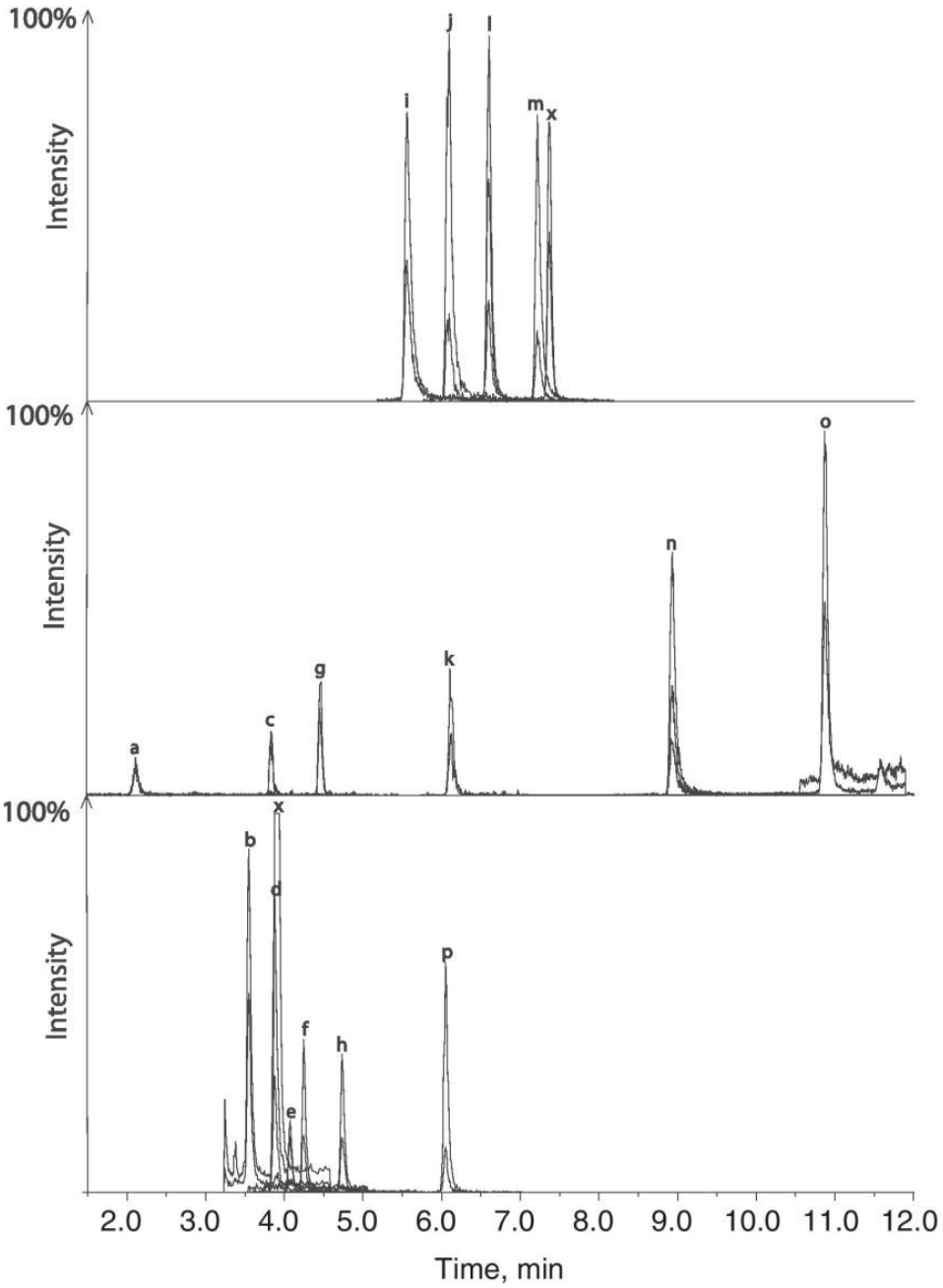


FIGURE 2 Chromatogram of a blank hair sample spiked with 50 pg/mg of each DOA. The chromatogram was split into the three groups (Top) cocaine, (Middle) opioids and (Bottom) amphetamines and methylphenidate for better visibility. Peaks are labeled in order of their RT. No smoothing was applied. ISTDs were omitted for clarity. (a) morphine; (b) amphetamine; (c) codeine; (d) methamphetamine; (e) MDA; (f) MDMA; (g) MAM; (h) MDE; (i) BE; (j) cocaine; (k) AcCo; (l) NorCoc; (m) CE; (n) EDDP; (o) MTD; (p) methylphenidate; (x) signals not belonging to a DOA

and Miller[17] were adapted and combined. The hair is pulverized and extracted for at least 4 hours with 1.5 mL ammonium formate buffer (200 nM, pH 5): methanol 1:1. The extract is centrifuged and the supernatant filtered using a syringe filter (polypropylene, 0.45 µm). The filtrate is evaporated and reconstituted in 0.5 mL water. A reduction of labor time of approximately 2 hours for a sample preparation of 10 samples for analysis of EtG and DOA is possible using the combined method. Additionally, the amount of used disposables can be slightly reduced. The method is not routinely used, as only a limited number of samples require an analysis of both substance groups. Instead, the method is used in special cases in which an analysis of both EtG and DOA is required and the amount of hair sample is too low for routine analysis while keeping a sample for a confirmation analysis. The validation parameters of the combined method are similar to the routine method parameters (not shown), meaning there is no loss of quality when using the combined method. The combined method is not more sensitive than other methods, with LOQs in the same range as other published methods for EtG[13,25] and DOA.[26,27]

The DOA extract was reconstituted in formate buffer after the SPE to ensure analyte stability for storage.[28] Ammonium formate buffer, which is used in the authors' routine extraction for DOA, cannot be used for extraction of the hair matrix in the method described here, as the buffer interferes with the SPE for EtG. Comparison of control samples prepared with the combined method to the routine methods shows no significant cocaine or MAM hydrolysis during sample workup without ammonium formate buffer.

In contrast to the generally used procedure of having 1 transition acting as quantifier and any further transitions used only as qualifiers, in this study all transitions except for the EtG transition (221.1 → 75.1) were used as quantifiers. Analysis results were calculated as the mean of all quantifiers. This was done when no transition showed clearly better results in the validation. The experience at the authors' lab shows that by using the mean of multiple transitions, the results are closer to the mean of all labs in round robin tests and the results of the control samples are more homogenous. This is presumably due to compensation of small fluctuations or interferences for single transitions.

Experiments done at our lab (not presented) show that the presented method can likely be expanded to include many benzodiazepines/z-substances. The benzodiazepines/z-substances can be extracted from hair using the same solvent mixture as used here and pass through the Oasis Max SPE cartridge in the same fraction as the DOA. The DOA extract was measured for benzodiazepines/z-substances using the routine method at our lab and showed similar results as the routine benzodiazepines/z-substances method in respect to LOD. However, as only a low number of benzodiazepine samples are tested at our lab, the method was not fully validated for these additional substances. Broadening the presented method to include the benzodiazepines could be interesting for laboratories with a larger number of samples. While the method was not tested for further substances, it is possible that it could be expanded to include other substances, for example tramadol, some antidepressants, neuroleptics, cathinones, or other new psychoactive substances (NPS).

4 | CONCLUSION

A method for the combined sample preparation of hair samples for measurement of EtG and common DOA from 1 aliquot of hair was developed and successfully validated for all substances. The presented method requires only 1 aliquot of 20 mg hair to measure EtG and a wide range of DOA. Especially in cases where only little hair is available, or many aliquots of hair are required, for example in segmental analysis with small segments, this method allows full analysis without resorting to lower sample amounts. Additionally, when analyzing for both EtG and DOA, sample preparation times can be slightly reduced. We expect that the presented approach of separating fractions on the SPE cartridge can be expanded to include further substances and aid in providing an optimal analysis even with limited sample amounts.

ORCID

Ulf Meier http://orcid.org/0000-0002-8284-9467
Eva Scheurer http://orcid.org/0000-0002-2742-0422

REFERENCES

1. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta*. 2006;370(1–2):17-49.
2. Kintz P. *Analytical and Practical Aspects of Drug Testing in Hair*. 1st ed. Boca Raton: CRC Press; 2007.
3. Kintz P, Salomone A, Vincenti M. *Hair Analysis in Clinical and Forensic Toxicology*. 1st ed. London: Academic Press; 2015.
4. Agius R, Nadulski T, Kahl HG, Dufaux B. Ethyl glucuronide in hair – A highly effective test for the monitoring of alcohol consumption. *Forensic Sci Int*. 2012;218(1–3):10-14.
5. Crunelle CL, Yegles M, van Nuijs AL, et al. Hair ethyl glucuronide levels as a marker for alcohol use and abuse: a review of the current state of the art. *Drug Alcohol Depend*. 2014;134:1-11.
6. Kissack JC, Bishop J, Roper AL. Ethylglucuronide as a biomarker for ethanol detection. *Pharmacotherapy*. 2008;28(6):769-781.
7. Pichini S, Gottardi M, Marchei E, et al. Rapid extraction, identification and quantification of drugs of abuse in hair by immunoassay and ultra-performance liquid chromatography tandem mass spectrometry. *cclm*. 2014;52(5):679-686.
8. Harkey MR. Anatomy and physiology of hair. *Forensic Sci Int*. 1993;63(1–3):9-18.
9. Society of Hair Testing. Recommendations for hair testing in forensic cases. *Forensic Sci Int*. 2004;145(2–3):83-84.
10. Cooper GA, Kronstrand R, Kintz P. Society of Hair T. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int*. 2012;218(1–3):20-24.
11. Schweizerische Gesellschaft für Rechtsmedizin. Bestimmung von Ethylglucuronid (EtG) in Haarproben. 2014; http://www.sgrm.ch/uploads/media/EtG_FINAL_2014.pdf. Accessed July 19, 2016.
12. Agius R. Utility of coloured hair for the detection of drugs and alcohol. *Drug Test Anal*. 2014;6(S1):110-119.
13. Binz TM, Baumgartner MR, Kraemer T. The influence of cleansing shampoos on ethyl glucuronide concentration in hair analyzed with an optimized and validated LC–MS/MS method. *Forensic Sci Int*. 2014;244:20-24.
14. Cappelle D, Neels H, Yegles M, et al. Gas chromatographic determination of ethyl glucuronide in hair: comparison between tandem mass spectrometry and single quadrupole mass spectrometry. *Forensic Sci Int*. 2015;249:20-24.

15. Pirro V, Di Corcia D, Seganti F, Salomone A, Vincenti M. Determination of ethyl glucuronide levels in hair for the assessment of alcohol abstinence. *Forensic Sci Int*. 2013;232(1–3):229-236.
16. Meier U, Briellmann T, Scheurer E, Dussy F. Distribution pattern of ethyl glucuronide and caffeine concentrations over the scalp of a single person in a forensic context. *Drug Test Anal*. 2017;9:1594-1603.
17. Miller EI, Wylie FM, Oliver JS. Simultaneous detection and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites, and opiates in hair by LC-ESI-MS-MS using a single extraction method. *J Anal Toxicol*. 2008;32(7):457-469.
18. Peters FT, Hartung M, Herbold M, Schmitt G, Daldrup T, Musshoff F. Anhang B zur Richtlinie der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen. Anforderungen and die Validierung von Analysemethoden. *Toxichem Krimtech*. 2009;76(3):185-208.
19. Agius R, Nadulski T, Kahl HG, et al. Validation of a headspace solid-phase microextraction-GC-MS/MS for the determination of ethyl glucuronide in hair according to forensic guidelines. *Forensic Sci Int*. 2010;196(1–3):3-9.
20. Salomone A, Baumgartner MR, Lombardo T, Alladio E, Di Corcia D, Vincenti M. Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: Comparison of positivity rates and appraisal of cut-off values. *Forensic Sci Int*. 2016;267:60-65.
21. Favretto D, Vogliardi S, Stocchero G, Nalesso A, Tucci M, Ferrara SD. High performance liquid chromatography-high resolution mass spectrometry and micropulverized extraction for the quantification of amphetamines, cocaine, opioids, benzodiazepines, antidepressants and hallucinogens in 2.5 mg hair samples. *J Chromatogr A*. 2011;1218(38):6583-6595.
22. Society of Hair Testing. Consensus for the Use of Alcohol Markers in Hair for Assessment of both Abstinence and Chronic Excessive Alcohol Consumption. 2016; http://www.soht.org/index.php/consensus. Accessed November 17, 2016.
23. Cordero R, Paterson S. Simultaneous quantification of opiates, amphetamines, cocaine and metabolites and diazepam and metabolite in a single hair sample using GC-MS. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;850(1–2):423-431.
24. Romolo FS, Rotolo MC, Palmi I, Pacifici R, Lopez A. Optimized conditions for simultaneous determination of opiates, cocaine and benzoylecgonine in hair samples by GC–MS. *Forensic Sci Int*. 2003;138(1–3):17-26.
25. Kronstrand R, Brinkhagen L, Nystrom FH. Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months. *Forensic Sci Int*. 2012;215(1–3):51-55.
26. Fernandez Mdel M, Di Fazio V, Wille SM, Kummer N, Samyn N. A quantitative, selective and fast ultra-high performance liquid chromatography tandem mass spectrometry method for the simultaneous analysis of 33 basic drugs in hair (amphetamines, cocaine, opiates, opioids and metabolites). *J Chromatogr B Analyt Technol Biomed Life Sci*. 2014;965:7-18.
27. Kim J, Ji D, Kang S, et al. Simultaneous determination of 18 abused opioids and metabolites in human hair using LC–MS/MS and illegal opioids abuse proven by hair analysis. *J Pharm Biomed Anal*. 2014;89:99-105.
28. Isenschmid DS, Levine BS, Caplan YH. A comprehensive study of the stability of cocaine and its metabolites. *J Anal Toxicol*. 1989;13(5):250-256.

How to cite this article: Meier U, Briellmann T, Scheurer E, Dussy F. Sample preparation method for the combined extraction of ethyl glucuronide and drugs of abuse in hair. *Drug Test Anal*. 2017;1–10. https://doi.org/10.1002/dta.2314

3.3. Project 3: Distribution Pattern of Common Drugs of Abuse, Ethyl Glucuronide, and Benzodiazepines in Hair Across the Scalp

About Project 3

This publication describes a thorough investigation of the concentration distribution patterns of EtG, DoA, and BZD-Z across the head for a collective of 14 persons. Additionally, the publication describes the measurement of the head skin perfusion and sweating rates across the scalp and compares them to the concentration distributions.

Authors: Ulf Meier, Flora College, Stephan Imfeld, Thomas Briellmann, Katja Mercer-Chalmers-Bender, Eva Scheurer, Franz Dussy

Contributions of Ulf Meier:

- Planning the study
- Writing all documents required for ethics committee
- Managing recruitment including writing/designing recruitment materials and distribution of materials
- Project management of the study including establishing collaboration with partners
- Conducting the study including taking the hair samples and conducting the sweat rate measurements as well as involvement in perfusion measurement.
- Conducting the sample preparation, measurements and data evaluation
- Writing the article

CRediT Statement:

Ulf Meier:	Conceptualization, formal analysis, investigation, visualization, methodology, project administration, writing – original draft
Flora College:	Investigation, writing – review & editing
Stephan Imfeld:	Investigation, writing – review & editing
Thomas Briellmann:	Conceptualization, writing – review & editing
Katja Bender:	Writing – review & editing
Eva Scheurer:	Conceptualization, supervision, writing – review & editing
Franz Dussy:	Conceptualization, supervision, writing – review & editing

Status: Published

Note: The supporting information is too extensive to be presented here. For all distribution patterns as well as sweat rate measurements please visit: https://onlinelibrary.wiley.com/doi/pdf/10.1002/dta.2679.

Received: 11 July 2019 | Revised: 5 August 2019 | Accepted: 5 August 2019

DOI: 10.1002/dta.2679

RESEARCH ARTICLE

WILEY

Distribution pattern of common drugs of abuse, ethyl glucuronide, and benzodiazepines in hair across the scalp

Ulf Meier[1] | Flora Colledge[2] | Stephan Imfeld[3] | Thomas Briellmann[1] | Katja Mercer-Chalmers-Bender[1] | Eva Scheurer[4] | Franz Dussy[1]

[1] Department of Forensic Chemistry and Toxicology, University of Basel Institute of Forensic Medicine, Basel, Switzerland

[2] Department of Sport, Exercise and Health, University of Basel, Basel, Switzerland

[3] Department of Angiology, University Hospital Basel, Basel, Switzerland

[4] University of Basel Institute of Forensic Medicine, Basel, Switzerland

Correspondence
Ulf Meier, University of Basel Institute of Forensic Medicine, Forensic Chemistry and Toxicology,Switzerland
Email: ulf.meier@bs.ch.

Abstract

While hair analysis is important and accepted in forensic applications, fundamental knowledge gaps still exist, exacerbated by a lack of knowledge of the incorporation mechanisms of substances into hair. The influence of the hair sampling location on the head on ethyl glucuronide (EtG) and cocaine concentrations was investigated by measuring the complete scalp hair of 14 (2 EtG, 4 cocaine, 8 both EtG and cocaine) study participants in a grid pattern for EtG, drugs of abuse, and benzodiazepines. Head skin perfusion and sweating rates were investigated to rationalize the concentration differences. For EtG, ratios between maximum and minimum concentrations on the scalp ranged from 2.5 to 7.1 (mean 4.4). For cocaine, the ratios ranged from 2.8 to 105 (mean 17.6). EtG concentrations were often highest at the vertex, but the distribution was strongly participant dependent. Cocaine and its metabolites showed the lowest concentrations at the vertex and the highest on the periphery, especially at the forehead. These differences led to hair from some head parts being clearly above conventional cut-offs and others clearly below. In addition to EtG and cocaine, the distributions of 24 other drugs of abuse and benzodiazepines/z-substances and metabolites are described. No clear pattern was observed for the head skin perfusion. Sweating rate measurements revealed higher sweating rates on the periphery of the haircut. Therefore, sweat could be a main incorporation route for cocaine. Concentration differences can lead to different interpretations depending on the sampling site. Therefore, the results are highly relevant for routine forensic hair analysis.

KEYWORDS

distribution, drugs of abuse, hair analysis, perfusion rates, sweating rates

1 | INTRODUCTION

Hair has become a popular matrix for forensic toxicological purposes, such as abstinence controls,[1] for example driving license regranting procedures,[2] workplace drug testing,[3,4] and child custody cases.[5-7] Hair testing has many benefits for both the examiner and the examinee. Advantages over traditional matrices such as blood and urine include longer windows of detection, easy and non-invasive sampling, sample stability, and easy storage. Using segmental hair analysis, information on exposure history based on the growth rate of the hair can be obtained.[8]

However, hair analysis also suffers from several pitfalls such as wash-out effects,[9,10] variable growth rates, and the dependence of basic substance incorporation on the melanin content of the hair.[11,12]

Many of these problems are exacerbated by the lack of a clear understanding of incorporation mechanisms into hair. A recent study involving the complete analysis of all scalp hair for ethyl glucuronide (EtG) and caffeine of a single test subject showed that concentrations of EtG and caffeine in hair depend on the sampling location on the head.[13] Factors of 3.0 and 10.6 were measured for EtG and caffeine, respectively, between the lowest and the highest concentrations.

The key limitation of that study was that it was only conducted with one person, and that no drugs of abuse (DoAs) or benzodiazepines/z-substances (BZD-Z) were examined. Therefore, in the current study, 14 test subjects who consumed alcohol and/or cocaine were examined. All head hair was collected in a grid pattern and analyzed for EtG, caffeine, DoA, BZD-Z, and common metabolites using liquid chromatography–tandem mass spectrometry (LC–MS/MS). As cocaine metabolite-to-parent ratios are often used to discriminate between contamination and consumption, these were also investigated.[14-16] Incorporation of substances from the blood stream into the growing hair and diffusion of substances from sweat or sebum into the hair are considered important incorporation mechanisms.[8,17] Therefore, relative head skin perfusion rates and sweating rates were measured to obtain a better understanding of the sampling-site-dependent concentration differences.

2 | MATERIALS AND METHODS

2.1 | Study design

The inclusion and exclusion criteria for study participation are shown in Table 1. Participant hair color and self-reported consumption behavior are shown in Table 2. Cocaine-consuming study participants reported either snorting or injecting cocaine. Smoking cocaine was not reported by any participant. The values of the participant of the previously conducted study were included (participant 0) to increase the number of study participants positive for EtG.[13] All study participants were male as no women could be enrolled. Before being included in the study, candidates were asked about their age, their health issues, and their alcohol, DoA, and medicine use. They were informed about the study and asked for their written consent. A hair sample was collected and analyzed for EtG and cocaine to determine suitability for study participation. The study involved two visits for the participants. During the first visit, the entire scalp hair was collected. On the second visit, the head was shaved and the perfusion and sweating rates were measured. Of the 13 (+participant 0) enrolled participants, 11 (participants 0, 4, and 8 missing) completed the perfusion measurements and 11 (participants 0, 4, and 5 missing) completed the sweating rate measurements. Missing perfusion measurements data were caused by technical difficulties with the measuring computer. Missing sweating rate measurements were caused by one participant having a mild asthma attack and another lacking the stamina to start sufficiently sweating from exercise. Approval of the ethics committee EKNZ (Ethikkommission der Nordwestschweiz) was granted (BASEC ID 2017–00628).

TABLE 1 Inclusion and exclusion criteria for participation

Inclusion Criteria	Exclusion Criteria
Chronic alcohol consumption ≥3 months and/or	Large bald spots
Chronic cocaine consumption ≥3 months Hair length ≥ 3 cm	Cosmetic treatment of hair Contraindication for sport
18 years or older	Insufficient German/English language skills to understand instructions
EtG and/or cocaine concentration above 7 pg/mg or 500 pg/mg, respectively.	

All head hair of the participants was collected. For this, sample areas of around 9 cm^2 were isolated and locks individually collected. A median of 79 (range 68 to 118) hair locks were collected per person. The corner points of each sample area were marked with a black marker on the head of the participants. After collection of all head hair, photos were taken to allow reconstruction of the sampling location. All strands were cut to a uniform length of 3 cm. For 10 of the 14 study participants, one lock in the vertex region was split into four smaller locks to examine the variability within one 9 cm^2 region in the vertex. This was done to test the reproducibility when sampling directly adjacent hair locks. Each hair lock was packaged in aluminum foil to protect it against light and stored at room temperature until analysis. Each hair lock was analyzed for EtG, caffeine, DoA, and BZD-Z, including typical metabolites.

The remaining hair stubbles (around 1–2 mm) were removed by wet shaving as the hair stubbles would interfere with the perfusion and sweating rate measurements. The relative perfusion rates were measured using a laser Doppler perfusion imager. For this, the participants lay face down. Before commencing with the measurement, the participants rested for around 10 minutes to adjust to the temperature in the room as heart rate and skin temperature can affect perfusion rates. Each sample area was scanned to allow a one-to-one assignment of hair concentrations to a perfusion value.

Sweating rates were measured using a cycling cap lined with water absorbing pads. Sweating was induced by cycling on an ergometer. The suitability of participants to participate was confirmed with an electrocardiogram prior to exercising. The exercise was set to be at a low-to-medium intensity. This was determined by the heart rate of the participants with the maximum allowed heart rate calculated as

$$HR_{max} = 211 - 0.64^{*}Age \quad (1)$$

with HR_{max} being the maximum allowed heart rate and *Age* being the age of the participant.[18] Participants were asked to cycle with a frequency between 80 and 90 rpm. The resistance of the ergometer was continually increased until participants said it was moderately straining and their heart rates reached 60%–70% of their maximum allowed heart rate. If the heart rate of participants exceeded the limit, the resistance of the ergometer was decreased until an acceptable level was reached.

TABLE 2 Hair color and self-declared alcohol, DoA, and BZD-Z consumption of study participants

#	Hair Color	Alcohol	DoA	BZD-Z
0	Brown	Approx. 35 g/d	None	None
1	Dark brown	Beer 1.5 L/week	Cocaine 3-4x/week Sevre-long® 1740 mg/d	Valium® 30 mg/d
2	Brown	Beer 1–1.5 L/week Sporadic vodka	Heroin 1 g/d Cocaine 0.5–1 g/week MDMA 1 month prior Sevre-long® 600 mg/d	Imovane® 7.5 mg/d
3	Brown graying	Beer 1 L/d	Methadone 60 mg/d Cocaine 3–4 x 0.5 g/week Heroin 3–4 x 0.2 g/week	Valium® 20 mg/d
4	Brown	Beer 1–1.5 L/d	Cocaine 1-2 g/d Heroin 2.5 g/d Methadone 200 mg/d Methylphenidate 60 mg/d	None
5	Black	Rare (ca. 1/3 months) but on those occasions much	Cocaine 2x 1–2 g/week Amphetamine sporadic	Temesta® 3x1mg/d
6	Brown	Beer 1–1.5 L/month	Cocaine 0.5–1 g/d Methadone 120 mg/d Heroin 1x0.4 g/month Amphetamine (2 months ago) up to 4 g/d Ritalin® 4x20mg/d	Stilnox® 2x12.5 mg/d Valium® sporadic Xanax® sporadic
7	Brown	Beer 1 L/week	Cocaine 2–3 months ago 0.25–0.5 g/week Amphetamine	Lorazepam sporadic
8	Brown graying	None	Cocaine 1–3 x 1-2 g/week Methadone 40 mg/d Ritalin® no dose declared	None
9	Brown graying	Beer 2–3 L/d	None	None
10	Brown graying	Sporadic “strong” consumption	Cocaine 2x(1/4)g/week Heroin occasionally Methadone 100 mg/d	Valium® on rare occasions
11	Dark brown graying	Beer 1 L/d	Cocaine 2–4 g/week Heroin 5–15 g/week Ritalin® 75 mg/d	None
12	Light brown	Beer 0.5–1 L/d	Cocaine 0.5 g/d Heroin occasionally 0.5 g Sevre-long® 600 mg/d	Occasionally Valium®
13	Black	Beer 0.5–1 L/d 0.5 L hard liquor/week	Cocaine, no dose declared Sevre-long® 920 mg/d Ritalin® no dose declared	Valium® 3-4x10 mg/week

Participants were asked to cycle for 20 minutes. The first 10 minutes of cycling were used to reach an appropriate heart rate and to induce sweating. During this time, participants cycled without a cap. For some participants, 10 minutes was not enough to start sweating and the time had to be increased, while for others it was necessary to shorten the time to 5 minutes as their stamina did not allow for a longer time. Afterwards, while participants continued to cycle, their head was thoroughly wiped dry with paper towels and the sweat cap placed on their head. The cap was worn for 10 minutes to collect the sweat. Afterwards, the sweat pads were quickly placed in sealable plastic bags.

2.2 | Chemicals and instrumentation

All reference and internal standards were supplied by Lipomed (Arlesheim, Switzerland), except brotizolam (LGC Standards GmbH, Wesel, Germany). An EtG stock solution at a concentration of 10 ng/µL in methanol was prepared. Stock mixes containing either caffeine, all DoA, or all BZD-Z standards at a concentration of 10 ng/µL in acetonitrile were prepared. Working standard solutions with various concentrations were prepared from the stock solutions as needed. An internal standard working mix containing EtG-D5, caffeine-D9, DoA, and BZD-Z internal standards was used, containing

0.05 ng/μL, 10 ng/μL, 2 ng/μL, and 1.25 ng/μL in acetonitrile, respectively. Standards and internal standards were stored at 4°C. LC–MS solvents water, acetonitrile, and methanol were obtained in analytical grade purity from Machery-Nagel AG (Oensingen, Switzerland). Formic acid puriss p.a. (98%), ammonium formate (≥ 99.0%), and ammonium hydroxide solution (25%) were obtained from Sigma-Aldrich (Buchs, Switzerland). 2-Propanol (≥ 99.5%) and acetone (≥ 99.5%) used for washing the hair were purchased from Roth (Arlesheim, Switzerland). Deionized water used for washing the hair was produced in-house.

3 | METHODS

3.1 | Hair sample preparation and analysis

Hair samples were prepared using a combined sample preparation method for EtG, DoA, BZD-Z, and caffeine followed by analysis with LC–MS^3 and LC–Multiple Reaction Monitoring (MRM). The BZD-Z that were included are presented in Table 3. The following DoA and metabolites were included: cocaine, benzoylecgonine, norcocaine, cocaethylene, morphine, 6-monoacetylmorphine, codeine, acetylcodeine, methadone, EDDP, amphetamine, methamphetamine, methylphenidate, MDMA, MDA, and MDE. Analysis was carried out using an Ultimate 3000 high performance liquid chromatograph (Thermo Fisher Scientific, Reinach, Switzerland) coupled to a 5500 QTrap triple quadrupole mass spectrometer (Sciex, Brugg, Switzerland). The method has previously been published,[19] but the BZD-Z and caffeine were not included. The BZD-Z were integrated into the method and fully validated according to the guidelines of the German speaking society of toxicology and forensic chemistry (GTFCh).[20] Caffeine was included in the method but was not fully validated. The linearity of caffeine was confirmed from 0.5 to 13 ng/mg and the limit of quantification (LOQ) was estimated from the calibration row as 0.5 ng/mg. The limit of detection (LOD) of caffeine was not determined.

Hair locks of around 50–100 mg were briefly (1–2 minutes) washed with 3 mL water, followed by 3 mL 2-propanol and finally 3 mL acetone. Strands were dried under a stream of nitrogen and cut into snippets of around 1 mm. Approximately 20 mg of the hair snippets were pulverized in a 2 mL Eppendorf tube equipped with two 5 mm stainless steel balls with an MM 200 ball mill (Retsch, Haan, Germany) operated at a frequency of 30 Hz for 7 minutes. To the powdered hair, 10 μL internal standard mix (0.5 ng EtG-D5, 100 ng caffeine-D9, 20 ng for each DoA, and 5 ng Brotizolam for the BZD-Z) were added. Each hair sample was extracted for 4 hours with 1.5 mL water:methanol 1:1 on an overhead shaker. Samples were centrifuged at 13 000 RPM (13793 g) for 10 minutes. EtG was separated

TABLE 3 Mass transitions (Q1; Q3), expected retention time (RT), declustering potential (DP), and collision energy (CE) for the BZD-Z. Table is ordered according to RT. Brotizolam was used as internal standard

BZD-Z	Q1 [Da]	Q3 [Da]	RT [min]	DP [V]	CE [V]
7-Aminoclonazepam	286.1	121.2/222.3/250.3	5.7	58	45/35/32
Zopiclone	389.0	345.1/217.0/245.0	5.9	55	15/45/25
7-Aminoflunitrazepam	284.2	256.2/227.2/135.1	7.0	100	30/35/45
Zolpidem	308.2	263.2/235.2/221.1	7.3	100	35/50/55
Midazolam	326.2	291.2/244.2/209.1	9.3	120	40/40/50
Bromazepam	316.1	209.2/290.1/209.2	9.5	80	40/30/40
Clonazepam	316.2	270.1/241.1/214.1	10.3	100	40/50/60
α-Hydroxymidazolam	342.2	324.1/297.1/203.1	10.4	120	30/40/40
Flunitrazepam	314.2	268.2/239.2/211.2	10.4	100	40/50/50
Clobazam	301.2	259.2/224.2/153.2	10.7	75	32/47/57
Oxazepam	287.2	269.1/241.1/231.1	11.0	80	22/35/35
Lorazepam	303.1	275.2/277.2	11.1	100	35/35
Alprazolam	309.2	281.2/274.2/255.0	11.1	100	40/40/40
Triazolam	343.1	315.1/308.2/239.1	11.3	90	40/40/60
Temazepam	301.1	283.1/255.1/228.1	11.4	65	20/35/35
N-Desalkylflurazepam	289.2	261.1/226.2/140.0	11.4	100	35/40/45
Lormetazepam	335.1	289.2/177.1/317.2	11.8	65	32/60/20
Nordazepam	271.2	208.2/165.1/140.1	11.9	110	40/40/40
Diazepam	285.2	222.1/193.1/154.1	12.3	110	40/50/40
IS-Brotizolam 1	393.1	314.2	11.6	80	35
IS-Brotizolam 2	395.1	314.2	11.6	80	35
IS-Brotizolam 3	397.1	316.1	11.6	80	35

from caffeine, DoA, and BZD-Z using a solid phase extraction (SPE) cartridge. For this, the supernatant was loaded onto an Oasis Max SPE cartridge (Waters, Baden-Dättwil, Switzerland), which had been conditioned with 2 mL methanol followed by 2 mL water. Caffeine, DoA, and BZD-Z are only partially retained on the SPE cartridge. Therefore, the fraction of the supernatant passing through the SPE cartridge was collected. The cartridge was washed with 2 mL MeOH and the wash solution containing the remaining caffeine, DoA, and BZD-Z was combined with the first fraction and set aside. The cartridge was further washed using 1 mL of water followed by 2 mL of MeOH. These two washing fractions were discarded. The cartridge was dried for 10 minutes under vacuum. EtG was eluted using 2 mL methanol with 2% formic acid. Both the caffeine/DoA/BZD-Z and EtG containing extracts were dried separately under a gentle nitrogen stream. The caffeine/DoA/BZD-Z extract was reconstituted in 0.5 mL ammonium formate buffer (200 nM, pH 5), while the EtG extract was reconstituted in 50 µL water.

BZD-Z were analyzed using LC–MRM. A Phenomenex Kinetex 50 x 2.1 mm 2.6 µm column (Brechbühler, Schlieren, Switzerland), protected by a Phenomenex KrudKatcher Ultra 0.5 µm 316 stainless steel depth filter pre-column (Brechbühler, Schlieren, Switzerland) was used for separation. A flow rate of 500 µL/min ammonium formate buffer (10mM ammonium formate, set to pH 3.4) with the following LC program was used for the gradient program: Starting with 90% (2 minute hold) to 40% at 12 minutes and 5% at 13 minutes adjusted to 100% with methanol. BZD-Z were measured with injection of 20 µL in positive APCI mode. The instrument was operated with a probe temperature (TEM) of 550°C, curtain gas (CUR) of 30 psi, a nebulizer gas (GS1) of 45 psi, a nebulizer current (NC) of 3 V, an ionspray voltage (IS) of 5500 V, an entrance potential (EP) of 10 V, and a collision cell exit potential of 12 V. The collision gas (CAD) was set to 8. Substance-specific instrument parameters and mass transitions are described in Table 3. Data acquisition was done using a scheduled MRM program with a target cycle time of 0.3 seconds.

The standard calibrated ranges for EtG, DoA, BZD-Z, and caffeine were 5–100 pg/mg, 50–5000 pg/mg, 5–500 pg/mg, and 2–12.5 ng/mg, respectively. In cases for which concentrations between the LOQ and the lowest calibrator were obtained, an additional calibrator at the LOQ was included. In some cases, the concentrations exceeded the linear range. In these cases, the injection volume was reduced (minimum 1 µL) and a calibration row with the corresponding concentrations was measured. If this was not sufficient, samples were diluted or re-prepared with lower amounts of hair.

3.2 | Perfusion measurement

Perfusion measurements were done with a laser Doppler perfusion imager PIM II (Perimed AB, Järfälla, Sweden) equipped with a 670 nm solid state laser. The scanning head was placed at a uniform distance of 20 cm from the skin. Scanning was done as perpendicular to the head surface as possible. This was important, as distance and angle can affect sensitivity of the measurement.[21,22] A maximum of four sample areas could be acquired simultaneously, due to the angle and distance requirements of the scanning head. The total time to scan the complete scalp was approximately 1.5 hours. The following software settings were used: image size 32x32 pixels, resolution medium, speed standard.

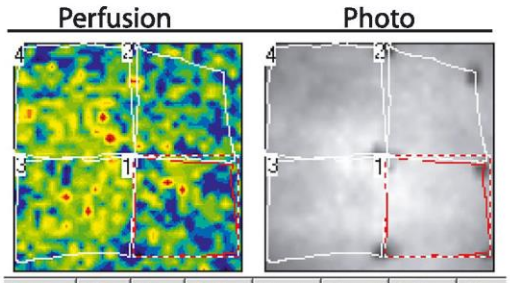
The output of the laser Doppler perfusion imager is Volts. This output cannot be correlated directly to the underlying flow rate and cannot be directly compared between participants, as the output is dependent on other factors such as lighting, skin color, etc. Instead, the outputs can only be compared within the subject. Therefore, the output is a relative perfusion measurement of each sample area compared to the other sample areas of that participant. For a review on laser Doppler methodology see Rajan et al.[23]

The laser Doppler perfusion imager performs single point scans in a raster. The mean value of all single point measurements (ca. 100–300 points) within a sample area was used for the evaluation of the perfusion. The mean was formed by manually marking the bounds of each sample area in the software. An example is shown in Figure 1.

In laser Doppler perfusion imaging, the biological zero (BZ) should be considered. The BZ is the change in frequency caused by the interaction of non-perfused tissue with the laser and has been attributed to the Brownian motion of molecules in the interstitial fluid causing a tissue and temperature dependent shift in the laser frequency.[22,24] It has therefore been proposed that the BZ should be subtracted from the obtained signal to be able to compare measurements. However, subtraction of the BZ is controversially discussed in the literature as subtraction can lead to an underestimation of the perfusion.[25] To investigate the BZ of scalp tissue, a small sample of scalp tissue obtained during a routine autopsy was heated with a water bath to be close to the temperature of the human skin surface of about 33°C.[26] The perfusion of the sample was measured ten times and the mean of the results used to estimate the BZ of scalp tissue. The standard deviation of the tissue measurements was used as an estimate of the repeatability of the measurements.

3.3 | Sweating rates

A cycling cap lined with strongly water absorbing pads (Airlaid Fabric 2205, Technical Absorbents Limited, Grimsby, UK) was used to measure the sweating rates. For this, the cycling cap (UV CAP, Vaude) was lined with support pads of 4x4 cm dimension. On top of these pads, smaller 3x3 cm measurement pads were attached using double-sided adhesive tape. Support and measuring pads were made of the same strongly water absorbing material. The measurement pads were stored in sealable plastic bags (Minigrip®, 50 mm x 75 mm polyethylene, Semadeni, Ostermundigen, Switzerland) to prevent contamination of the pad or evaporation of sweat from the pad. Sweating rates were measured gravimetrically by comparing the weight of the measurement pad before and after being applied to the head of the participant. The support pad served multiple purposes. First, it prevented sweat from the surrounding areas reaching the



Item	Min	Max	Std.d...	Mean	Change	% Ch...	Sites
ROI 1	0.20	1.54	0.18	0.68	ref	ref	185
ROI 2	0.32	1.26	0.15	0.68	-0.01	-1.18	179
ROI 3	0.31	1.48	0.19	0.76	0.07	10.73	229
ROI 4	0.31	1.64	0.20	0.76	0.07	10.70	237

FIGURE 1 Example illustrating the fashion of manual integration of the sample areas to get mean perfusion values. (A) Red areas show areas of higher perfusion. (B) Black-and-white photograph of the measured area. (C) Example output of the perfusion of the four integrated areas [Colour figure can be viewed at wileyonlinelibrary.com]

measurement pad. Second, sewing it on using a two-point attachment system allowed the cap to expand and contract without the pads falling off the cap or bending while allowing rapid removal of the measurement pads and use for every head shape and size. Rapid removal of the measurement pads was important to prevent evaporation before placing them in the sealable bag. The pads were bagged immediately after completion of the exercise with bagging taking approximately 5–10 minutes. To avoid a systematic error, the sweat pads were bagged in a random order. An example cap with a schematic representation is shown in Figure 2. On each cap, 39 support and measuring pads were attached. The pads were attached to the cap in three concentric rings with 13, 12, and 8 pads for the outside (ring 1), middle (ring 2), and inner ring (ring 3), respectively. At the top of the cap (at the position of the vertex), one larger support pad was attached to which the six remaining measurements pads were taped.

The difference in weight between the dry and wet pad was used to calculate the sweating rate as

$$SR = \frac{W_{wet} - W_{dry}}{t_{cycling} * A_{pad}} \quad (2)$$

with SR being the sweating rate in mg ^{1} min^{-1} cm^{-2}, W_{wet} being the weight of the wet pad, W_{dry} being the weight of the dry pad, $t_{cycling}$ being the cycling time while wearing the cap and A_{pad} being the area of the pad.

In a pre-experiment, the evaporation rate of water from sweat pads was investigated to estimate the weight lost to evaporation during the time between exercise and bagging the pads. For this 50 µL (a), 100 µL (b), 200 µL (c), and 500 µL (d) distilled water was added to three pads each. The pads were placed openly on a table top at room temperature (22°C). The weight of each pad was measured at 0.5, 1, 2, 3, 4, and 5 minutes to track the evaporation. The evaporation for the sweat pads was linear with a mean of 5.3 ± 0.1% (a), 4.7 ± 0.3% (b), 4.0 ± 0.2% (c), and 2.9 ± 0.3% (d) of the applied amount evaporated after 5 minutes.

3.4 | Data analysis and software

Evaluation of the chromatograms of all analytes was carried out using the software MultiQuant (version 3.0.2, Sciex, Brugg, Switzerland). Spearman correlations were calculated using the statistics software STATA (version 12.1, StataCorp LLC, College Station, TX, USA). For sample areas with concentrations between the LOD and the LOQ,

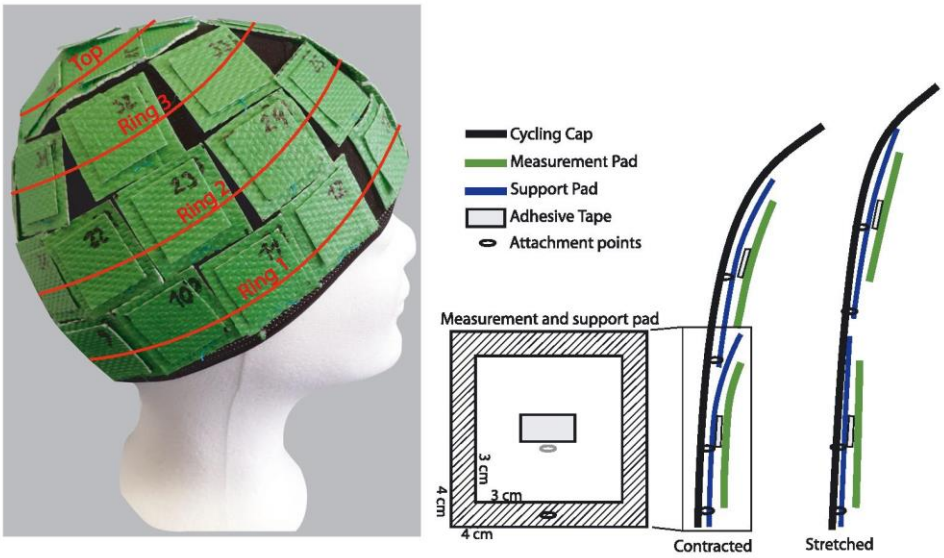


FIGURE 2 (A)Example sweat cap turned inside out. For sweat measurement 39 support and measurement pads were used. The sweat pads were ordered in three concentric rings. At the top of the head in the vertex region one larger support pad was attached, to which six measurement pads were taped. (B) Schematic construction plan of the sweat cap showing the functioning principle of the attachment points and the support and measurement pads [Colour figure can be viewed at wileyonlinelibrary.com]

approximate concentrations were used for the purpose of correlation. Sample areas with non-detectable concentrations were treated as missing for correlation. All other calculations were done using Microsoft Excel 2010 (Redmond, WA, USA). All graphs were made using GraphPad Prism 5 (La Jolla, CA, USA).

As the head shape and the haircut of every participant were different, an adapted number of samples were collected. The exact locations of sample areas were not the same across the participants. For the comparison of results between participants, the heads were divided into the six anatomical regions, namely forehead, frontal, vertex, parietal, occipital, and temporal, as shown in Figure 3. The observed substance concentrations in the hair varied considerably from participant to participant. Therefore, normalized concentrations of the head regions were calculated. To normalize the concentrations, mean concentrations of all samples within each of the head regions were calculated. The head region mean concentrations were normalized to the mean concentration of the vertex head region (vertex set to 1) to give relative head region concentrations for each participant. Additionally, the division into anatomical regions facilitates the transfer of the results of this study to routine work, as exact sampling location will not be documented during routine sampling. The division of the heads into regions was done with photos after completion of the study.

Ratios between maximum and minimum concentration were used to characterize the extent of concentration differences. For some study participants, individual hair strands showed substance concentrations below the LOQ or the substance was undetectable. If the lowest concentration was below the LOQ, the ratio was calculated as maximum concentration found on the head divided by the LOQ of that substance. If substances could not be detected in some hair sample areas, the ratio was calculated as maximum concentration found on the head divided by the LOD of that substance.

To classify the distribution behavior of substances and to allow a comparison of the distribution of the substances, each substance was classified according to how "cocaine-like" their distributions were. For this, for every cocaine positive participant, the Spearman correlations between the cocaine concentration and the concentration of every other detected substance for each sample area of that participant were calculated. Substances were classified as moderately to strongly cocaine-like (coefficient: 1.0–0.6), as fairly cocaine-like (coefficient: 0.6–0.2), as non-cocaine-like (coefficient: 0.2 – −0.2), as fairly reverse-cocaine-like (coefficient: −0.2 – −0.6), or as moderately to strongly reverse-cocaine-like (coefficient: −0.6 – −1.0). These categories are arbitrary but follow a common rule of thumb.[27]

4 | RESULTS

As the focus of the study was EtG and cocaine (+metabolites), the results concerning these substances are presented and discussed more extensively. All measurement results (hair analysis, perfusion, sweating rates) of all participants are available in the Supporting Information (Data S1 and Data S2).

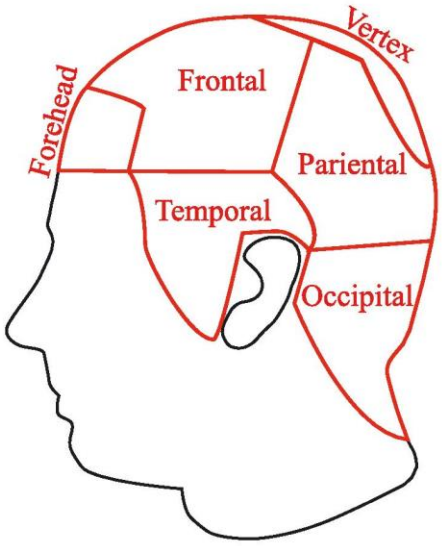


FIGURE 3 Classification scheme of the head into the six anatomical regions forehead, frontal, temporal, parietal, occipital, and vertex [Colour figure can be viewed at wileyonlinelibrary.com]

4.1 | Hair analysis

The method was successfully validated for all BZD-Z. The validation parameters of the BZD-Z are shown in Table 4. For the validation parameters of EtG and DoA see Meier et al.[19]

Hair of 14 participants was analyzed for EtG, caffeine, DoA, and BZD-Z yielding distribution patterns of 29 substances.

4.2 | EtG

The distribution of EtG values is shown in Table 5. The ratios between maximum and minimum concentrations found within the samples taken from the head of the individual participants are between 2.5 and 7.1 with a mean ratio of all participants of 4.4.

The shape of the distribution pattern of the participants is displayed in Figure 4A as a projection onto the head for participant 10, and two-dimensionally for all participants in Figure 5. The shape was not uniform for all participants. Many participants showed higher concentrations of EtG at the vertex and toward the forehead while lower concentrations were often found toward the neck. Others showed more erratic distribution patterns with spots of higher or lower concentrations. Many participants showed EtG concentration differences between the left and right side of the head. These differences were especially pronounced for participants 4 and 9. The deviation of the distribution patterns are better visible as box plots of the concentrations obtained for each anatomical region presented in Figure 6. Participants showed maximum concentrations in different anatomical regions and often presented considerable variation also

TABLE 4 Validation parameters for the BZD-Z (concentration levels: Low: 20 pg/mg; mid: 250 pg/mg; high: 500 pg/mg). Linearity was given for all substances from LOQ-2500 pg/mg (except 7-aminoflunitrazepam, diazepam, zolpidem, and flunitrazepam from 5 to 2500 pg/mg)

	LOD [pg/mg]	LOQ [pg/mg]	Accuracy Bias [%]			Intra-day Precision CV [%]			Inter-day Precision CV [%]			Matrix Effect [%]		Recovery [%]	
Substance			Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	High	Low	High
7-Aminoclonazepam	5	10	4.1	0.0	0.1	5.0	6.8	4.8	6.3	9.1	10	77 ± 8	81 ± 8	83 ± 6	77 ± 5
Zopiclon	5	10	−5.1	3.5	1.6	7.2	7.5	4.4	9.0	9.5	12	101 ± 7	95 ± 3	70 ± 7	72 ± 9
7-Aminoflunitrazepam	2.5	2.5	3.3	1.6	1.2	6.2	6.6	3.5	7.0	9.7	9.4	86 ± 11	88 ± 6	88 ± 10	78 ± 4
Zolpidem	2.5	2.5	−0.8	−1.0	−0.8	5.0	3.1	3.5	5.1	4.4	6.1	101 ± 5	101 ± 3	80 ± 6	83 ± 5
Midazolam	5	10	−2.0	−0.5	1.2	5.5	2.4	3.2	5.3	4.4	5.5	108 ± 9	102 ± 6	78 ± 5	82 ± 3
Bromazepam	10	25	3.3	3.5	3.7	5.6	3.9	3.5	4.3	5.1	5.6	94 ± 6	97 ± 3	73 ± 7	75 ± 7
Clonazepam	2.5	10	−5.3	−1.6	2.1	6.1	4.0	3.5	8.9	5.0	3.9	82 ± 7	86 ± 6	78 ± 7	83 ± 8
α-Hydroxymidazolam	5	10	−4.3	−4.8	−4.4	4.8	2.2	2.5	6.1	2.7	3.7	100 ± 10	97 ± 4	82 ± 9	82 ± 4
Flunitrazepam	2.5	2.5	6.7	4.8	4.6	4.8	3.3	2.8	5.7	4.5	5.1	93 ± 9	96 ± 4	82 ± 4	82 ± 3
Clobazam	5	5	2.7	1.7	−0.4	4.3	2.5	3.1	4.3	5.6	6.5	97 ± 8	96 ± 4	83 ± 8	83 ± 2
Oxazepam	5	10	−5.1	−8.6	−6.0	5.5	2.9	3.8	7.3	3.9	5.3	116 ± 14	105 ± 6	73 ± 11	80 ± 6
Lorazepam	5	10	−1.3	−0.9	−0.1	4.9	3.5	2.9	7.9	3.5	2.9	97 ± 14	101 ± 6	82 ± 10	80 ± 6
Alprazolam	5	10	2.9	3.0	1.7	6.0	3.9	3.1	7.4	5.0	4.0	104 ± 17	99 ± 6	75 ± 19	83 ± 5
Triazolam	2.5	5	0.7	−2.7	−1.7	6.5	3.4	4.2	5.9	3.8	5.7	100 ± 7	100 ± 4	83 ± 8	86 ± 4
Temazepam	5	10	−2.4	−2.1	−2.5	4.0	3.7	2.7	6.6	5.1	5.0	91 ± 12	96 ± 4	81 ± 9	82 ± 5
N-Desalkylflurazepam	5	10	−2.1	−1.1	1.2	4.9	4.6	2.5	7.2	5.4	3.6	84 ± 13	88 ± 5	82 ± 8	83 ± 7
Lormetazepam	2.5	5	−2.7	−3.9	−4.0	3.9	3.9	3.7	5.2	5.1	5.6	99 ± 15	97 ± 3	89 ± 15	82 ± 4
Nordazepam	5	10	−5.6	−3.2	0.7	5.8	4.3	4.5	8.3	7.0	6.4	83 ± 9	87 ± 6	81 ± 11	80 ± 8
Diazepam	2.5	2.5	−3.7	−1.0	0.9	4.2	3.8	3.4	5.9	4.9	3.8	89 ± 8	90 ± 4	79 ± 5	82 ± 6

within the anatomical regions. The mean of the relative concentrations of all participants was marginally the highest at the vertex followed in descending order by the forehead and the frontal region, the parietal region, the temporal region, and finally the occipital region.

For seven participants, the EtG concentration variation within one sample area of the vertex was tested by splitting the area into four subareas. The results are presented in Table 6. For most participants, the variation within one sample area was small and in the range of what can be expected from measurement precision. Participant 9 showed fairly high variation. For this participant, the samples were taken from a sample area that was located on a strong concentration gradient. The concentrations of the individual subareas were comparable to the concentrations of samples adjacent to that area.

4.3 | Cocaine and metabolites

The distributions of cocaine and metabolite values are shown in Table 7. The mean ratios between maximum and minimum concentrations found within the samples from each participant for cocaine and metabolites are between 4.1 for cocaethylene and 17.6 for cocaine.

The shape of the distribution pattern is shown in Figure 4B for cocaine as a projection onto the head for participant 10 and two-

TABLE 5 Minimum, maximum, and median EtG concentrations, ratio between maximum and minimum concentration, and the number of samples for each participant (N)

Participant #	0	2	3	4	5	9	10	11	12	13
Min [pg/mg]	6.8	14.5	7.6	1.7*	3.1	173	6.2	3.5	6.8	n.d.
Max [pg/mg]	20.2	35.8	47.8	9.3	9.7	1190	30.4	17.8	16.8	7.1
Median [pg/mg]	13.7	27.2	20.8	4.7	5.4	551	17.0	12.2	9.7	4.0
Ratio	3.0	2.5	6.3	3.1	3.1	6.9	4.9	5.1	2.5	7.1†
N	104	76	81	95	76	82	71	68	70	69

The value marked with an asterisk is an approximate value as it is below the LOQ of 3 pg/mg. The ratio was calculated with minimum = 3.0 (LOQ). The ratio marked with a dagger was calculated with min = LOD = 1.0.

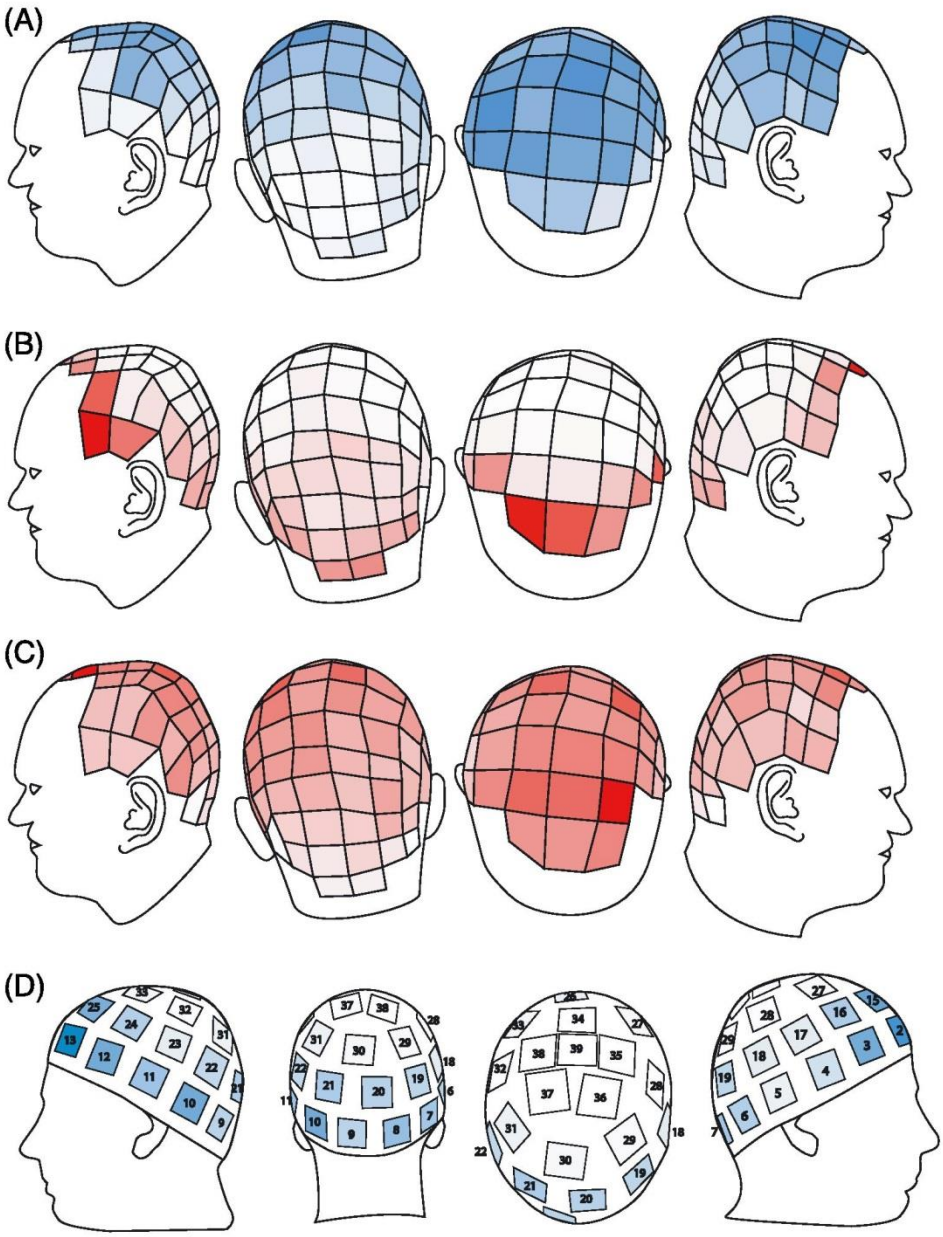


FIGURE 4 A, EtG; B, cocaine; C, perfusion; and D, sweating rate distribution of participant 10. The sweating rates are displayed on a model head, as it was not possible to precisely allocate the sweat pads to the head region of the individual participants [Colour figure can be viewed at wileyonlinelibrary.com]

dimensionally for all participants in Figure 7. Benzoylecgonine, norcocaine, and cocaethylene showed the same distribution pattern as cocaine. The distribution pattern of cocaine was very similar for all participants with the exception of participant 13 as visually assessed from the distributions data depicted in Figure 7 and from the assignment to the six head regions in Figure 8. Concentrations were highest at the forehead and generally higher on the edges of the haircut. The lowest concentrations were found in the area of the vertex or adjacent to the vertex. For participant 1, the highest concentrations were observed at the neck. In this case however, hair could not be sampled at the forehead and all the way along the periphery at the sides, as the participant had partially shaved his head in this region and the hair was not sufficiently long, in order to be comparable with hair segments analyzed from other participants. Participant 4 showed large differences in concentrations between the right and left side of the head. These differences are responsible for this participant's very high concentration ratios.

As presented in Figure 8, after splitting into the anatomical regions, the forehead generally showed the highest concentrations, followed by the temporal, frontal and occipital, parietal, and finally the vertex region. The same pattern was observed for benzoylecgonine, norcocaine and cocaethylene.

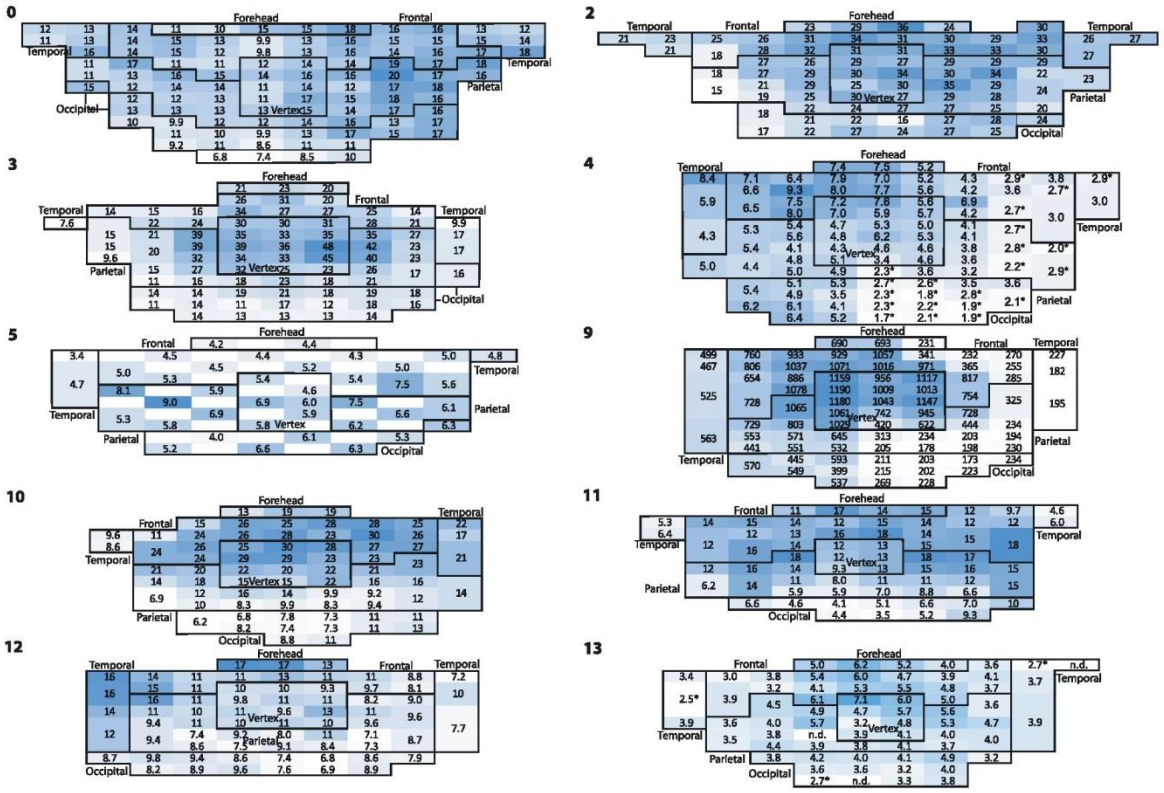


FIGURE 5 Distribution patterns of the 10 EtG positive study participants. Darker colors show higher concentrations. Colors are scaled intra-individually. Graphs are read as follows: Center = vertex, top = forehead; bottom = neck; top left = area at the left temple and vice versa. The transitions between the head regions are marked with a thick border. Concentrations are given in pg/mg. Values marked with an asterisk are below the LOQ and therefore approximate values [Colour figure can be viewed at wileyonlinelibrary.com]

The split into four smaller subareas for homogeneity testing was done for nine cocaine-positive study participants. As shown in Table 8, cocaine concentrations usually did not vary much within a sample area. In cases for which higher differences were observed within one sample area, a strong concentration gradient was present between the sample area and the surrounding areas.

While the shape of the distribution was nearly identical for cocaine and its metabolites, the extent of the distribution was substance dependent with cocaine showing the highest differences across the head followed by benzoylecgonine, norcocaine, and finally cocaethylene. This difference in extent of distribution caused the ratios between parent and metabolite concentrations to be sampling-site dependent. Sites with higher cocaine concentrations showed lower metabolite to parent ratios. Results of the metabolite to parent ratios are shown in Table 9.

4.4 | Other substances and distribution comparison

The distribution pattern of 24 other substances was also characterized. The ratio between maximum and minimum concentrations of the individual substances, the mean ration, and the number of participants positive for that substance are shown in Figure 9. Many substances regularly showed considerable differences across the head. The extent of these differences varied considerably inter-individually. The mean distribution of all participants across the six head regions normalized to the vertex posterior is exemplarily shown for opioids and diazepam (+ metabolites) in Figure 10.

For many substances, higher concentrations were found toward the edges of the haircut. While cocaine showed this pattern for all but one participant, the behavior of the other substances was not this regular, as participant-dependent distribution extent and pattern was observed. Substances were characterized according to how cocaine-like their distribution patterns were using Spearman correlation. The coefficient of every correlation for all participants and substances with the classification is shown in Figure 11. Benzoylecgonine, norcocaine, 6-monoacetylmorphine, and acetylcodeine always show moderately to strongly cocaine-like distributions. Most other substances show a more participant-dependent behavior, but the mean of correlation coefficient of all substances except EDDP, zopiclone, and EtG show fairly cocaine-like behavior. EtG is the only substance with a mean correlation coefficient with a fairly reverse-cocaine-like behavior.

4.5 | Perfusion measurements

To estimate the biological zero of scalp tissue, ten repeated measurements of a small scalp tissue sample were done. The mean value of

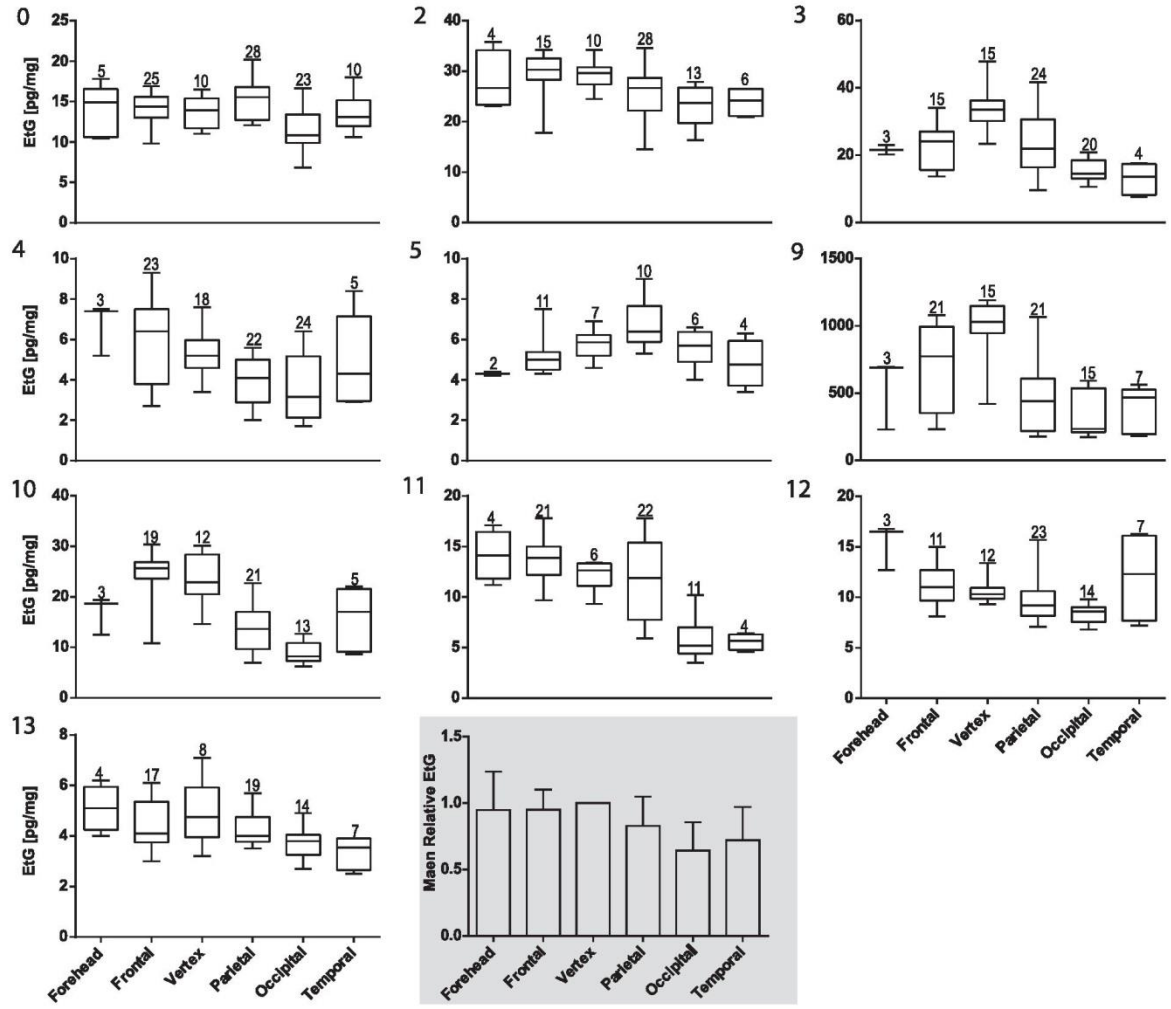


FIGURE 6 Box plots of the distribution patterns of the individual EtG positive study participants. The code of the study participant is shown at the top left of each graph. The whiskers show the minimum and maximum concentrations within a head region, the center line the median and the box the interquartile range between 25th and 75th percentiles. The number of hair sample areas assigned to each head region is shown above the top whisker. The mean relative (vertex = 1) EtG concentrations of the head regions of all participants (grey background; error bars show the standard deviation)

TABLE 6 The variation of the EtG concentrations obtained from four subareas within one sample area, the mean of the concentrations, the standard deviation (StD), and the coefficient of variation (%CV). Concentration values are given as pg/mg

Participant #	4	5	9	10	11	12	13
1. Value	4.6	6.0	622	15.6	15.3	13.4	4.8
2. Value	4.8	6.4	354	15.0	12.7	9.3	4.8
3. Value	4.6	5.3	611	13.0	16.6	10.1	3.5
4. Value	4.7	6.9	427	9.3	14.0	10.6	4.2
Mean	4.7	6.2	504	13.2	14.7	10.9	4.3
StD	0.1	0.7	134	2.8	1.7	1.8	0.6
%cv	2.0	11.0	26.6	21.5	11.5	16.4	14.3

the biological zero was 0.43 (±0.06) V with a coefficient of variation of 15%.

The head skin perfusion of participants was measured. Perfusion results could be obtained for 11 study participants. The distributions of the perfusion values without correction for BZ are shown in Table 10. In general, the perfusion across the scalp did not show clear patterns across participants. An exception to this was at the start of the hairline on the neck, where the perfusion values were lower than for the rest of the head. Occasionally, individual areas with considerably higher perfusion than the surrounding areas were observed. The distribution of the perfusion of participant 10 is shown exemplary in Figure 4C.

Subtracting the BZ massively increases the relative differences between sample areas. After subtraction, the low perfusion values at

TABLE 7 Minimum, maximum, and median cocaine, benzoylecgonine, norcocaine, and cocaethylene concentrations, ratios between maximum and minimum concentrations, and mean ratio

	Cocaine				Benzoylecgonine				Norcocaine				Cocaethylene			
#	Min	Max	Median	Ratio	Min	Max	Median	Ratio	Min	Max	Median	Ratio	Min	Max	Median	Ratio
1	4810	36673	15395	7.6	1505	7306	4246	4.9	181	469	266	2.6	27	67	40	2.5
2	5884	58347	12456	9.9	735	4813	1547	6.6	102	616	213	6.0	122	340	181	2.8
3	12167	91045	31010	7.5	4125	21844	12238	5.3	401	1672	771	4.2	1283	5535	2745	4.3
4	16122	1692461	110919	105	5997	233984	29854	39.0	630	8321	1568	13.2	36	303	69	8.4
5	28171	78589	41782	2.8	3757	10561	5356	2.8	418	796	558	1.9	234	378	283	1.6
6	17444	160510	47445	9.2	3068	15026	7474	4.9	538	2978	1134	5.5	6.5	32	12	4.9
7	312	4773	818	15.3	94	1428	265	15.2	9.4	45	18	4.8	-	-	-	-
8	9311	54313	18057	5.8	2365	8304	3709	3.5	415	1014	615	2.4	n.d.	22	13	2.2*
10	6706	172091	31713	25.7	1175	17259	5841	14.7	141	1912	400	13.6	736	6102	1726	8.3
11	24958	118571	51401	4.8	3299	13107	6390	4.0	965	2427	1671	2.5	141	336	231	2.4
12	35427	477798	113427	13.5	13423	65458	25736	4.9	450	4299	1109	9.5	619	3193	1082	5.2
13	55623	233524	99226	4.2	8964	24514	13283	2.7	733	2374	1253	3.2	1058	2652	1865	2.5
Mean				17.6				9.0				5.8				4.1

Concentrations are given as pg/mg. (*) Some hair samples were below LOD. Ratio calculated as max/LOD (=10 pg/mg)

the neck were less than two standard deviations (as determined by the repeated scalp tissue measurements) different from zero for 7 of 11 participants and considered non-significantly different from zero. This was considered unlikely and probably caused by the underestimation of the perfusion when the BZ is subtracted. Therefore, the non-corrected values were used.

Spearman correlation analysis of cocaine or EtG concentrations with the perfusion was done for participants for whom perfusion data were available. The p-value and Spearman rho are shown for each correlation in Table 11. A significant correlation was found in 4 of 8 cases for EtG and in 5 of 10 cases for cocaine. In cases with statistically significant correlations, the correlation coefficient was positive for EtG and negative for cocaine in every case with the strength of the relationship being weak to moderate.

4.6 | Sweating rates

Sweating rate measurements could be obtained for 11 study participants. A projection of the mean sweating rates of participant 10 onto a head is shown in Figure 4D. Figure 12 shows the mean sweating rates of the circularly arranged pads for each participant and the mean of all participants. Generally, sweating rates were highest on the outmost ring (ring 1, Figure 2) and decreased toward the vertex (top, Figure 2). Sweating rates also decreased from the front toward the back of the head. This decrease was especially pronounced on the periphery of the haircut leading to similar sweating rates for rings 1 and 2 at the back of the neck. The differences in sweating rates between the rings were quite large, with ratios between rings 1 and 2, 1 and 3, and 1 and top of 1.5, 2.7, and 8.2, respectively for the mean sweating rates of the rings. The sweating rates were assigned to the anatomical head regions. The mean sweating rates of all participants was highest at the forehead (2.24 mg ^{1} cm^{-2} min^{-1}), followed by the temporal (1.12 mg ^{1} cm^{-2} min^{-1}), frontal (0.98 mg ^{1} cm^{-2} min^{-1}), parietal (0.68 mg ^{1} cm^{-2} min^{-1}), occipital (0.67 mg ^{1} cm^{-2} min^{-1}), and finally the vertex region (0.23 mg ^{1} cm^{-2} min^{-1}). The relative sweating rates normalized to the vertex and the corresponding cocaine and metabolite concentrations are shown in Figure 8.

There was no significant correlation between the relative concentrations of EtG or cocaine of the individual head regions and the relative sweating rates of that head region, ie, participants with larger concentration differences did not necessarily have larger sweating rate differences (non-significant Spearman correlation [$p > 0.05$], not shown).

5 | DISCUSSION

5.1 | Distribution of substances, perfusion, and sweating rates

Large concentration differences depending on sampling site were found for both EtG and for cocaine and its metabolites. Cocaine and its metabolites showed a clear distribution with higher concentrations at the periphery of the hair-cut for all participants except for one. EtG on the other hand showed a very different distribution which was less well defined and more participant dependent. For many participants, EtG showed a distribution pattern which was opposed to that of cocaine, as illustrated in Figure 11 by the often negative Spearman correlation coefficient of EtG with cocaine. As the distributions of

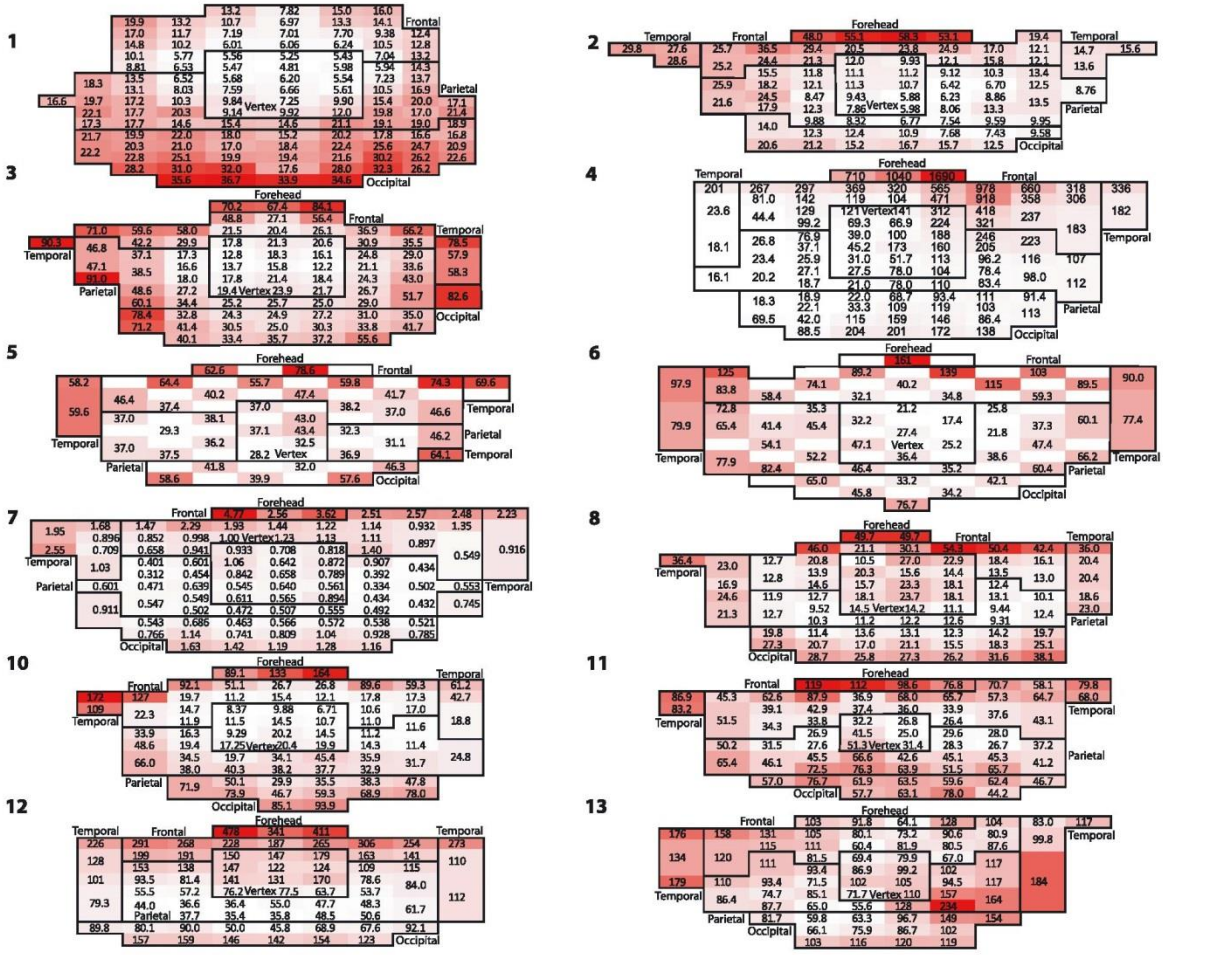


FIGURE 7 Cocaine distribution patterns of the 12 cocaine-positive participants. Darker colors show higher concentrations. Colors are scaled intra-individually. Graphs are read as follows: Center = vertex, top = forehead; bottom = neck; top left = area at the left temple and vice versa. The transitions between the head regions are marked with a thick border. Concentrations are given in ng/mg [Colour figure can be viewed at wileyonlinelibrary.com]

cocaine and EtG are often opposed, they likely differ fundamentally in the way they are incorporated into the hair with respect to the sampling location. Interestingly, most substances showed a behavior that was moderately to strongly cocaine-like. Furthermore, the data suggest that fairly small changes in the chemical structure can strongly impact the distribution, as can be seen from the extent of differences (Figure 9) and from how cocaine-like morphine and codeine are compared with 6-monoactelymorphin and acetylcodeine (Figure 11). The acetylated substances show more cocaine-like and more extreme concentration differences. As 6-monoacetylmorphine and acetylcodeine show more extensive differences in their distribution than their non-acetylated counterparts, lipophilicity might play an important role. However, it could also be caused by differences in pharmacokinetics for example, or by a combination of factors.

The perfusion across the head did not follow a clear pattern and only the neck regularly showed lower perfusion rates. However, this is most likely an artefact of the experimental setup as participants' heads were tilted slightly up while lying face down to facilitate measuring, thus compressing the blood vessels in this area.

Spearman correlations of the perfusion values with the EtG or cocaine concentrations were done for each participant. As the Spearman rho of significant correlations was always positive for EtG and negative for cocaine, this indicates that a higher perfusion is correlated to higher EtG concentrations and lower cocaine concentrations. However, the strength of the correlations was only moderate and the correlations were only significant in half of the cases. This is an indication that while the perfusion might play a minor role in causing the distribution patterns, it seems that there are other factors more strongly affecting the distribution, causing the correlations to be only moderately strong or non-significant for many participants.

Sweating rates were highest on the forehead. The sweating rates decreased from front to back and from the periphery to the center of the head. The sweating rates observed in this study were generally lower than those measured in a study from Machado-Moreira et al.[28] This is especially true for the vertex region for which they obtained about five-fold greater sweating rates. This can be explained by differences in the study setup. Machado-Moreira used water heated full body suits at 46°C and a mean exercised duration of 56 minutes which

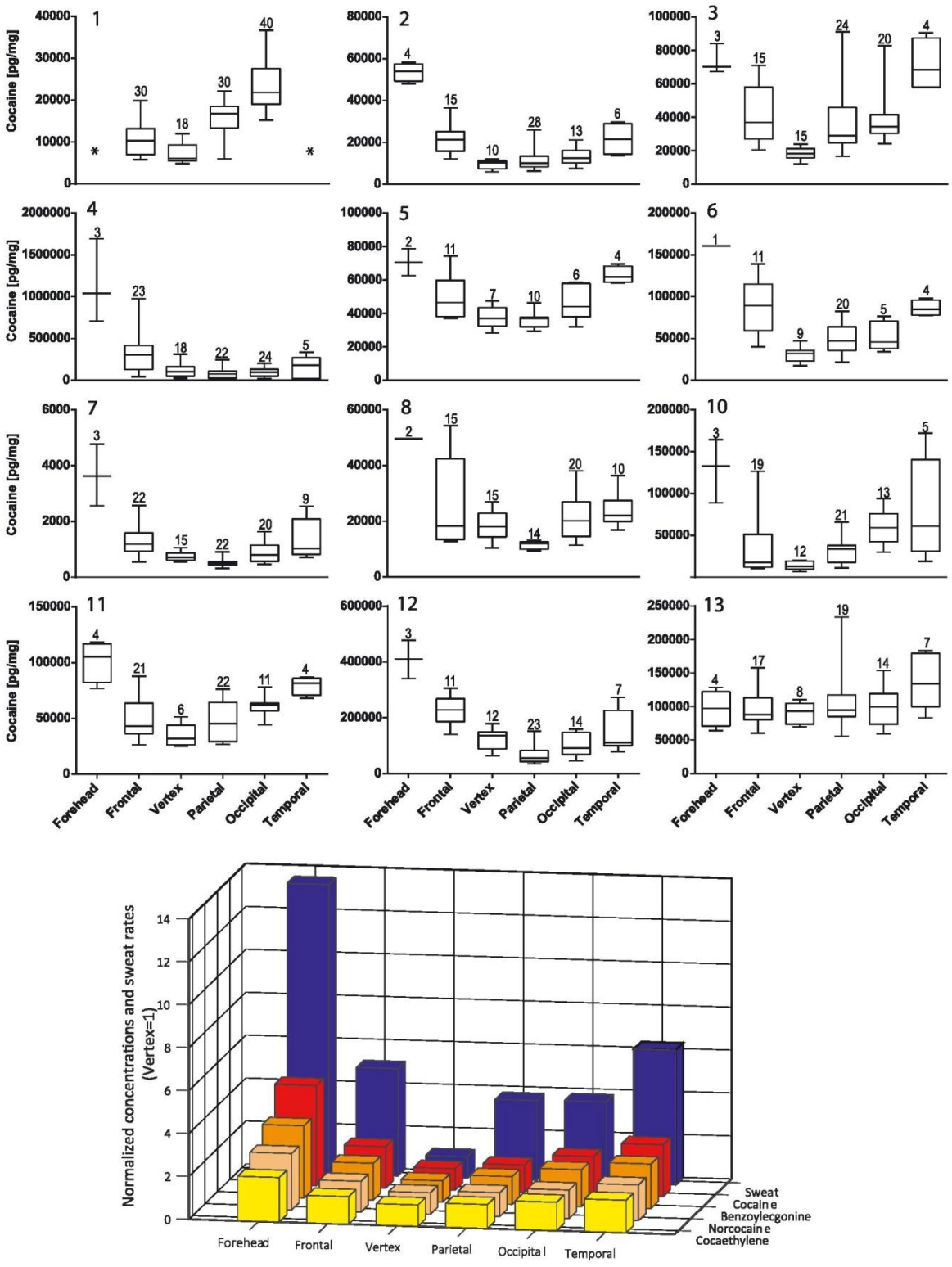


FIGURE 8 (A) Box plots of the distribution patterns of the individual cocaine-positive study participants according to the head region. The code of the study participant is shown at the top left of each graph. The whiskers show the minimum and maximum concentrations within a head region, the center line the median and the box the interquartile range between 25th and 75th percentiles. The number of samples areas assigned to each head region is shown above the top whisker. Concentrations are given in pg/mg. (*) For participant 1, no samples in the forehead or temporal region could be collected. (B) The mean relative (vertex=1) concentrations of cocaine, benzoylecgonine, norcocaine, and cocaethylene, and relative sweating rates of the head regions of all participants [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 8 Variation of the cocaine concentrations obtained from four subareas within one sample area, the mean of the concentrations, the standard deviation (StD), and the coefficient of variation (%CV). Concentrations are given as pg/mg

Participant #	4	5	6	7	8	10	11	12	13
1. Value	112753	43418	25152	561	12284	19671	28303	47654	105459
2. Value	91209	34174	33055	628	11286	23593	34485	142215	124420
3. Value	107401	39965	22228	374	12699	35934	28401	161415	107402
4. Value	96786	33564	22319	410	12734	34761	25043	103917	89837
Mean	102037	37780	25689	493	12251	28490	29058	113800	106780
StD	9805	4738	5095	121	675	8093	3940	50158	14146
%cv	9.6	12.5	19.8	24.5	5.5	28.4	13.6	44.1	13.2

TABLE 9 Minimum, maximum, and median cocaine metabolite-to-parent ratios of the individual participants (#)

	Benzoylecgonine/Cocaine		Norcocaine/Cocaine		Cocaethylene/Cocaine	
#	Min-max[%]	Median[%]	Min-max[%]	Median[%]	Min-max[%]	Median[%]
1	18–35	28	0.7–4.1	1.8	0.10–0.65	0.26
2	6–16	12	0.2–5.7	1.5	0.27–4.64	1.3
3	22–53	37	1.5–3.9	2.4	3.7–12.5	8.7
4	14–44	27	0.5–3.9	1.4	0.02–0.25	0.06
5	10–16	13	0.9–1.6	1.3	0.39–0.85	0.68
6	9–19	15	1.7–3.5	2.4	0.02–0.05	0.03
7	20–132	30	0.8–3.2	2.3	-	-
8	15–27	22	1.6–5.9	3.4	-	-
10	8–25	17	0.8–2.6	1.4	2.0–13.3	5.9
11	3–46	12	1.9–4.8	3.2	0.19–0.82	0.47
12	14–41	23	0.7–1.5	1.1	0.52–2.1	1.1
13	8–24	14	0.8–1.6	1.3	0.75–2.6	2.0

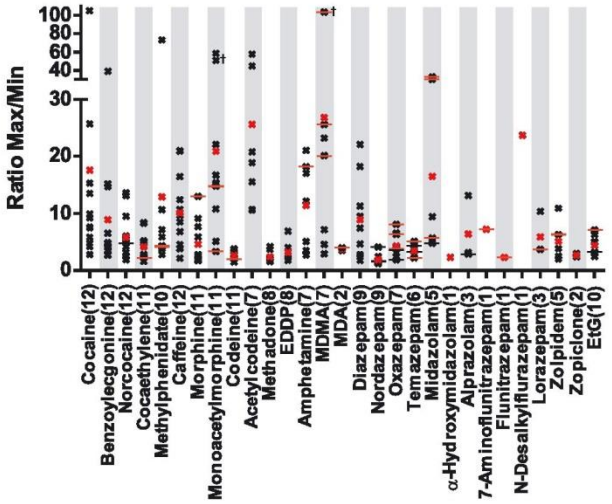


FIGURE 9 Ratio between maximum and minimum concentration for every observed substance is shown as a black cross. The mean ratio for each substance is shown as a red cross. For each substance, the number of participants whose hair was positive for that substance is shown after the substance name in parentheses. For participants with concentrations below the LOQ, the corresponding ratios are labeled with a black bar through the cross. For participants with hair regions below the LOD, the corresponding ratios are labeled with a red bar. The high ratios of 6-monoacetylmorphine (participant 1) and MDMA (participant 5) marked with a dagger are the result of two and one sample areas with high concentrations, respectively. A second analysis of those hair strands confirmed the results. They are likely the result of a contamination at that specific spot. Removing these high values gives ratios of 8.1 and 7.5 for 6-monoacetylmorphine and MDMA, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

is three times as long as in this study. It is plausible that the vertex sweat glands are slower to react to stimulation and that the equilibration time of 10 minutes used in this study did not fully activate them as regional differences in eccrine sweat gland activation as well as exercise duration dependent activation have been reported.[29,30] However, the employed exercise durations of around 20 minutes of medium intensity in this study are likely a better representation of the daily life exercise done by the study participants and therefore more relevant for this study.

Due to the experimental setup, the hair sample areas were not exactly the same as the sweating rate measurement areas. Therefore, a direct correlation of the results with the concentration distributions is not possible. However, assigning the sweat measurements to the six anatomical head regions allows a comparison of the sweating rates with the concentrations profiles. As shown in Figure 8, the distribution of relative sweating rates was similar to the concentration distribution of cocaine and cocaine metabolites. This indicates that the observed concentration differences are possibly caused by differences in sweating and that a significant portion of cocaine incorporation might take place via the sweat. As EtG often showed a reverse-cocaine-like behavior, EtG might in contrast be washed out by sweating. Additionally, the EtG that is excreted via sweat might not be incorporated into the hair, as EtG does not bind to melanin.[12] To be incorporated into the hair via sweat, the substance must first be able to enter the sweat via the blood and then be able to diffuse into the hair from the sweat. If the differences in sweating rates are a reason for the substance-dependent concentration differences observed across the scalp, it follows that substances with different distribution patterns must vary in one of these two steps. Therefore, it is likely that substances which

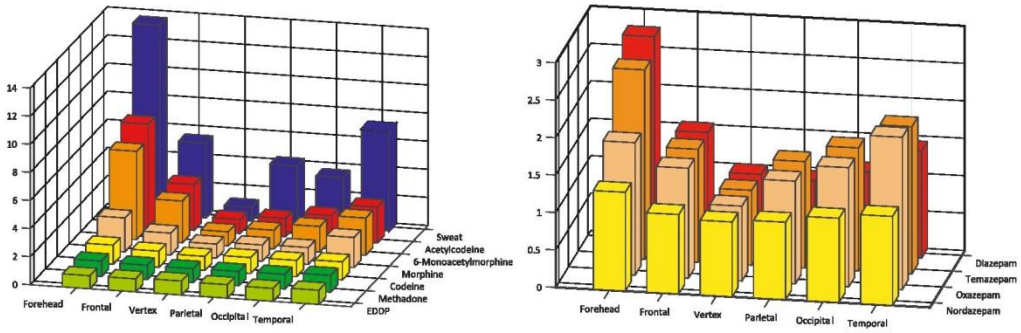


FIGURE 10 Mean relative (vertex=1) concentrations of (A) the opioids and (B) diazepam and its metabolites of the head zones of all participants [Colour figure can be viewed at wileyonlinelibrary.com]

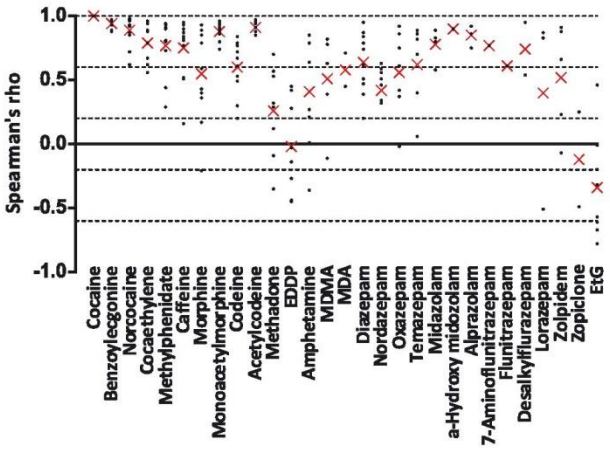


FIGURE 11 Spearman correlation coefficients of cocaine against every other substance for cocaine-positive participants. Each dot represents the correlation coefficient of one participant. The cross shows the mean coefficient of all participants. The dashed lines represent the borders of the classification (1 – 0.6: Moderately to strongly cocaine-like; 0.6 – 0.2: Fairly cocaine-like; 02 – -0.2: Non-cocaine-like; -0.2 – -0.6: Fairly reverse cocainelike; -0.6 – -1: Moderately to strongly reverse cocaine-like) [Colour figure can be viewed at wileyonlinelibrary.com]

show higher concentrations in peripheral regions are either better able to enter the sweat and/or better able to enter the hair from the sweat. Other possible reasons for the observed concentration differences, for example head region dependent hair melanin concentrations, hair length, sebum production rates, and hair porosity were not considered in this study. For some substances, such as morphine, the extent of differences was highly participant dependent. Influencing factors could be differences in pH of sweat, hair structure, co-consumption of other substances, differences in metabolism, or amount of consumed substance.

The non-significant correlation between relative sweating rates and relative concentration differences of the individual head regions can be explained by varying situations of the participants concerning sweat-inducing activities in the time period after drug consumption. Additionally, the sweat cap collected the sweat immediately as excreted, while normally the sweat would on the one hand run from the top to the periphery of the head and on the other hand be manually transferred from one point of the scalp to another.

While many participants showed slight concentration differences between the left and right sides of the head, participants 4 and 9 showed more pronounced laterally non-symmetric distributions. During the interview, participant 4 stated that he sweats much more on the right side of his body. Unfortunately, sweating rate data for this participant was not obtained. For this participant, much higher concentrations of many substances, for example cocaine, were observed on the right side than on the left side. EtG showed an opposed behavior. Unilateral sweating on the whole or parts of the body has been described in medical literature and can be caused by Harlequin syndrome,[31] Ross syndrome[32] or unknown mechanisms,[33] for example. However, in this study no detailed medical history was obtained. In the case of participant 9, a clear difference was seen in EtG concentrations on the right and left side of the head with higher concentrations on the left side. The sweating rate measurement of this participant yielded about three times higher sweating rates on the right side of the head than on the left. Again, this aligns with EtG showing a reverse behavior to the sweating rates.

TABLE 10 Minimum, maximum, and median perfusion value as well as the ratio between maximum and minimum perfusion values without subtraction of the BZ

Participant #	1	2	3	5	6	7	9	10	11	12	13
Min [V]	0.40	0.42	0.45	0.40	0.58	0.58	0.79	0.48	0.48	0.58	0.48
Max [V]	0.86	0.81	1.26	0.82	0.96	1.06	1.51	1.05	1.22	1.13	1.15
Median [V]	0.68	0.68	0.77	0.59	0.72	0.82	1.13	0.72	0.78	0.86	0.83
Ratio	2.2	1.9	2.8	2.1	1.7	1.8	1.9	2.2	2.5	2.0	2.4

TABLE 11 Results of the Spearman correlation of perfusion values with EtG or cocaine concentrations. Shown are the p-values, the Spearman rho, and the number of observations for each participant. Values marked in bold show significant ($p \leq 0.05$) correlations

Participant #	1	2	3	5	6	7	9	10	11	12	13
P-value EtG	N/A	0.20	**<0.00**	0.39	N/A	N/A	**<0.00**	**<0.00**	0.14	0.13	**0.02**
Rho EtG	N/A	−0.15	**0.39**	−0.14	N/A	N/A	**0.43**	**0.64**	0.18	−0.18	**0.30**
p-value cocaine	**<0.00**	0.11	**<0.00**	0.75	0.74	**<0.00**	N/A	**<0.00**	0.16	**<0.00**	0.93
Rho cocaine	**−0.32**	0.19	**−0.50**	−0.05	−0.05	**−0.46**	N/A	**−0.47**	0.17	**−0.60**	−0.01
N	117	74	80	39	49	89	82	72	67	70	67

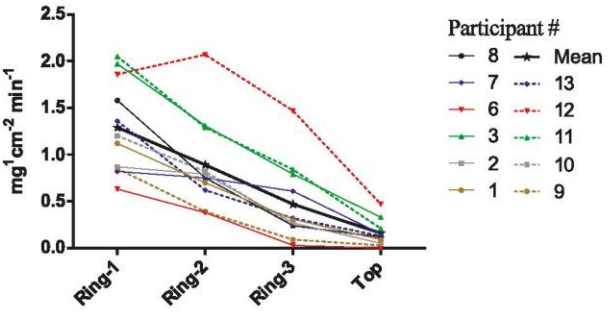


FIGURE 12 Sweating rates obtained for ring 1, ring 2, ring 3, and the top of the sweat cap for each participant. The mean of the sweating rates of all participants for each ring is shown as a bold line [Colour figure can be viewed at wileyonlinelibrary.com]

As another source of the observed distribution external contamination of the hair by running cocaine powder contaminated fingers through the hair, for example, has to be discussed. However, this is not supported by the observed results. The distribution is very regular across many participants. It seems unlikely that each participant would contaminate his hair in exactly the same way. Furthermore, the metabolites benzoylecgonine, norcocaine, and cocaethylene showed the same regional distribution as cocaine. While these have been occasionally found in street cocaine, the concentrations are usually low and do not explain the observed pattern.[34-36] Lastly, each hair strand was washed before being analyzed. Therefore, substances adsorbed to the surface of the hair should be removed to a large extent and mainly the incorporated fraction should subsequently be measured.

5.2 | Limitations

The measurement with the laser Doppler perfusion imager necessitated shaving the participants' heads. While the utmost care was taken to avoid any damage to the skin during shaving, in some cases mild skin rashes occurred. These rashes caused higher perfusion rates in the affected areas. The head was closely visually inspected and any rashes or other skin damage (eg, pimples, scars) were photographically documented. These areas were excluded from analysis of the perfusion rates. However, it is possible that occasionally skin irritations were not detected leading to erroneously high perfusion rate measurements in some areas.

The penetration depth of the laser used in this study is about 0.3–0.4 mm.[37] The cutaneous microcirculation is organized in two horizontal layers, the upper being the subpapillary and the lower the dermal plexus.[38] From the subpapillary plexus, papillary loops rise to just below the surface of the skin. The used method is mainly measuring the blood flow in the papillary loops or of the subpapillary plexus while the hair roots are supplied from the dermal plexus. The two systems are connected by a system of vertical blood vessels. We therefore assume that the perfusion rates measured in the upper layer are representative of the perfusion in the lower layer.

The method used for sweating rate measurements in this study has not been validated. However, very similar methods using sweat absorbent pads (same material as in this study) have been validated and compared with the ventilated capsule sweat measuring method.[39] The results were found to be comparable between the two methods. The only notable change in methodology in this study is the attachment of many pads to a cycling cap to simultaneously measure sweating on the entire head.

5.3 | Relevance of results for forensic routine work

The large differences in concentrations depending on the sampling site are highly significant for routine forensic hair analysis. The observed differences led to different interpretations depending on the sampling location. Especially for EtG, the large differences can have a substantial effect on the evaluation of a hair analysis result. In the investigated study collective, one participant (participant 10) showed concentrations below the Society of Hair Testing (SoHT) proposed abstinence cut-off of 7 pg/mg to above the proposed chronic excessive drinking cut-off of 30 pg/mg, and many participants showed concentrations in both the abstinent and social drinking, or social drinking and excessive drinking ranges. While this is more problematic for EtG as it has two cut-offs used for judgment of drinking behavior, it is of course also a problem for the DoA and BZD-Z with proposed cut-offs for reporting non-adherence of abstinence. Cocaine showed very high variations across the head. This can lead to situations in which some parts of the head will be clearly above the cut-off and others clearly below. This is the case for participant 7 of this study, whose cocaine concentrations in the hair range from 312 pg/mg to 4773 pg/mg, so from clearly below to more than nine-fold above the SoHT's proposed cut-off of 500 pg/mg. In forensic cases, these differences could cause different estimations of the consumption behavior or could change

the interpretation when judging how plausible a stated consumption behavior is. The results underline that the assessment of consumption behavior from hair results should be viewed with caution. During monitoring, sampling from different head regions could lead to false interpretations in regard to changing consumption behavior. Secondly, the differences can cause problems in confirmation analysis. If the second lock was taken from a different location than the first lock, large concentration differences can be expected leading to doubts about the validity of the measurements or raising suspicions of a sample mix-up. Thirdly, the sampling site dependent parent-metabolite ratios can lead to a different interpretation of the results. Parent-metabolite ratios are often used to differentiate between consumption and external contamination.[36,40,41] As there are different recommendations for discriminating between consumption and external contamination based on parent-metabolite ratios, each lab will have to independently judge if the presented results are problematic for their interpretation. Finally, the observed differences might in part be responsible for the oftentimes weak correlation between consumption and head hair concentrations.[42-46] If hair was sampled from different locations during such studies, a part of the variation might arise from sampling-site-dependent differences. Therefore, the sampling location should be strictly controlled in the design of studies.

6 | CONCLUSION AND SAMPLING RECOMMENDATION

In conclusion, large differences for EtG, DoA, and BZD-Z were found depending on the location of sampling on the head. Concentrations were typically higher on the periphery of the haircut for most substances. A notable exception to this was EtG, which often showed the highest concentrations at the center in the vertex region. Sweating rate measurements of the study participants revealed higher exercise-induced sweating rates at the periphery of the haircut, especially at the forehead. As this aligns with the concentration distribution of most DoA and some BZD-Z, we suspect that differences in sweating are the main cause of the observed concentration differences. Only a weak to moderate relationship between head skin perfusion and hair concentration differences was observed.

For several reasons and as demonstrated by the shown data, the current recommendation of sampling in the vertex posterior by several professional bodies should be adhered to. First, the established cut-offs and recommended parent-metabolite ratios are based on the vertex posterior. The vertex posterior is the best described area as most research has been done on hair from this region. If sampling has to be done from other head regions, application of recommended cut-offs and drawing conclusion from hair testing results, especially for EtG, requires caution. Besides the fact that the applied decontamination strategy as well as the analytical method can have a strong influence on the result, sampling from other head regions than the vertex posterior may add further uncertainty and can cause false statements. Second, the vertex posterior generally showed higher EtG concentrations. As EtG is a low concentrated marker,[47-49] it is reasonable to sample from the location with the highest concentrations in order to refute a self-declared alcohol abstinence. However, there might be cases for which EtG is not important and a high sensitivity for DoA or BZD is required. In these cases, it would be reasonable to sample hair from the periphery of the haircut, ideally from the forehead. Before fully recommending this approach however, studies on the distribution of external contamination on the head should be conducted. The forehead might be especially vulnerable to external contamination, as hair close to the forehead is often touched with the hands. Finally, the concentration gradients were usually least steep in the vertex area. This is reflected in the relatively homogeneous results of the within-sample area repeat measurements. We therefore also support the recommendation of the European Workplace Drug Testing Society (EWDTS) to sample two or more directly adjacent hair locks.[4] As the vertex posterior generally showed a concentration plateau, sampling adjacent strands from this area will likely yield comparable concentrations.

ACKNOWLEDGEMENTS

We would like to sincerely thank lab assistant Bettina Butscher for her valuable help in assisting during the hair sampling and for preparing many hair strands for analysis. We would further like to thank Dr Sebastian Ludyga for evaluating the ECGs prior to the exercise and advising in planning the exercise duration and intensity.

ORCID

Ulf Meier https://orcid.org/0000-0002-8284-9467
Flora Colledge https://orcid.org/0000-0001-5406-7276
Katja Mercer-Chalmers-Bender https://orcid.org/0000-0002-3843-9065
Eva Scheurer https://orcid.org/0000-0002-2742-0422

REFERENCES

1. Cooper GA. Hair testing is taking root. *Ann Clin Biochem*. 2011;48(Pt 6):516-530.
2. Polla M, Stramesi C, Pichini S, Palmi I, Vignali C, Dall'Olio G. Hair testing is superior to urine to disclose cocaine consumption in driver's licence regranting. *Forensic Sci Int*. 2009;189(1–3):e41-e43.
3. Caplan YH, Goldberger BA. Alternative specimens for workplace drug testing. *J Anal Toxicol*. 2001;25(5):396-399.
4. Salomone A, Tsanaclis L, Agius R, Kintz P, Baumgartner MR. European guidelines for workplace drug and alcohol testing in hair. *Drug Test Anal*. 2016;8(10):996-1004.
5. Couper FJ, Chopra K, Pierre-Louis ML. Fatal methadone intoxication in an infant. *Forensic Sci Int*. 2005;153(1):71-73.
6. Pragst F, Krumbiegel F, Thurmann D, et al. Hair analysis of more than 140 families with drug consuming parents. Comparison between hair results from adults and their children. *Forensic Sci Int*. 2019;297:161-170.
7. Pichini S, Garcia-Algar O, Alvarez AT, et al. Pediatric exposure to drugs of abuse by hair testing: monitoring 15 years of evolution in Spain. *Int J Environ Res Public Health*. 2014;11(8):8267-8275.
8. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta*. 2006;370(1–2):17-49.

9. Luginbuhl M, Nussbaumer S, Weinmann W. Decrease of ethyl glucuronide concentrations in hair after exposure to chlorinated swimming pool water. *Drug Test Anal*. 2018;10(4):689-693.
10. Jurado C, Kintz P, Menendez M, Repetto M. Influence of the cosmetic treatment of hair on drug testing. *Int J Leg Med*. 1997;110(3):159-163.
11. Rothe M, Pragst F, Thor S, Hunger J. Effect of pigmentation on the drug deposition in hair of grey-haired subjects. *Forensic Sci Int*. 1997;84(1–3):53-60.
12. Appenzeller BM, Schuman M, Yegles M, Wennig R. Ethyl glucuronide concentration in hair is not influenced by pigmentation. *Alcohol Alcohol*. 2007;42(4):326-327.
13. Meier U, Briellmann T, Scheurer E, Dussy F. Distribution pattern of ethyl glucuronide and caffeine concentrations over the scalp of a single person in a forensic context. *Drug Test Anal*. 2017;9(10):1594-1603.
14. Cone EJ, Yousefnejad D, Darwin WD, Maguire T. Testing human hair for drugs of abuse. II. Identification of unique cocaine metabolites in hair of drug abusers and evaluation of decontamination procedures. *J Anal Toxicol*. 1991;15(5):250-255.
15. Cooper GA, Kronstrand R, Kintz P, Society of Hair Testing. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int*. 2012;218(1–3):20-24.
16. Han E, Park Y, Yang W, et al. The study of metabolite-to-parent drug ratios of methamphetamine and methylenedioxymethamphetamine in hair. *Forensic Sci Int*. 2006;161(2–3):124-129.
17. Henderson GL. Mechanisms of drug incorporation into hair. *Forensic Sci Int*. 1993;63(1):19-29.
18. Nes BM, Janszky I, Wisloff U, Stoylen A, Karlsen T. Age-predicted maximal heart rate in healthy subjects: the HUNT fitness study. *Scand J Med Sci Sports*. 2013;23(6):697-704.
19. Meier U, Briellmann T, Scheurer E, Dussy F. Sample preparation method for the combined extraction of ethyl glucuronide and drugs of abuse in hair. *Drug Test Anal*. 2018;10(4):701-710.
20. Peters FT, Hartung M, Herbold M, Schmitt G, Daldrup T, Musshoff F. Anhang B zur Richtlinie der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen. Anforderungen and die Validierung von Analysemethoden. *Toxichem Krimtech*. 2009;76(3):185-208.
21. Droog EJ, Steenbergen W, Sjoberg F. Measurement of depth of burns by laser Doppler perfusion imaging. *Burns*. 2001;27(6):561-568.
22. Kernick DP, Shore AC. Characteristics of laser Doppler perfusion imaging in vitro and in vivo. *Physiol Meas*. 2000;21(2):333-340.
23. Rajan V, Varghese B, van Leeuwen TG, Steenbergen W. Review of methodological developments in laser Doppler flowmetry. *Lasers Med Sci*. 2009;24(2):269-283.
24. Kernick DP, Tooke JE, Shore AC. The biological zero signal in laser Doppler fluximetry - origins and practical implications. *Pflugers Arch*. 1999;437(4):624-631.
25. Zhong J, Seifalian AM, Salerud GE, Nilsson GE. A mathematical analysis on the biological zero problem in laser Doppler flowmetry. *IEEE Trans Biomed Eng*. 1998;45(3):354-364.
26. Montgomery LD, Williams BA. Effect of ambient temperature on the thermal profile of the human forearm, hand, and fingers. *Ann Biomed Eng*. 1976;4(3):209-219.
27. Akoglu H. User's guide to correlation coefficients. *Turk J Emerg Med*. 2018;18(3):91-93.
28. Machado-Moreira CA, Wilmink F, Meijer A, Mekjavic IB, Taylor NA. Local differences in sweat secretion from the head during rest and exercise in the heat. *Eur J Appl Physiol*. 2008;104(2):257-264.
29. Kondo N, Takano S, Aoki K, Shibasaki M, Tominaga H, Inoue Y. Regional differences in the effect of exercise intensity on thermoregulatory sweating and cutaneous vasodilation. *Acta Physiol Scand*. 1998;164(1):71-78.
30. Kondo N, Shibasaki M, Aoki K, Koga S, Inoue Y, Crandall CG. Function of human eccrine sweat glands during dynamic exercise and passive heat stress. *J Appl Physiol (1985)*. 2001;90(5):1877-1881.
31. Lance JW, Drummond PD, Gandevia SC, Morris JG. Harlequin syndrome: the sudden onset of unilateral flushing and sweating. *J Neurol Neurosurg Psychiatry*. 1988;51(5):635-642.
32. Mullaaziz D, Kaptanoglu AF, Eker A. Hypohidrosis or hyperhidrosis? Ross syndrome. *Dermatologica Sinica*. 2016;34(3):141-143.
33. Kocyigit P, Akay B, Vural S, Akbostanci M, Bostanci S. Unilateral hyperhidrosis with accompan ying contralateral anhidrosis. 2009;34(8):e544-e546.
34. Hoelzle C, Scheufler F, Uhl M, Sachs H, Thieme D. Application of discriminant analysis to differentiate between incorporation of cocaine and its congeners into hair and contamination. *Forensic Sci Int*. 2008;176(1):13-18.
35. Casale JF, Klein RFX. Illicit production of cocaine. *Forensic Sci Rev*. 1993;5(2):95-107.
36. Ropero-Miller JD, Huestis MA, Stout PR. Cocaine analytes in human hair: evaluation of concentration ratios in different cocaine sources, drug-user populations and surface-contaminated specimens. *J Anal Toxicol*. 2012;36(6):390-398.
37. Fredriksson I, Larsson M, Stromberg T. Measurement depth and volume in laser Doppler flowmetry. *Microvasc Res*. 2009;78(1):4-13.
38. Braverman IM. The cutaneous microcirculation. *J Investig Dermatol Symp Proc*. 2000;5(1):3-9.
39. Morris NB, Cramer MN, Hodder SG, Havenith G, Jay O. A comparison between the technical absorbent and ventilated capsule methods for measuring local sweat rate. *J Appl Physiol (1985)*. 2013;114(6):816-823.
40. Madry MM, Rust KY, Guglielmello R, Baumgartner MR, Kraemer T. Metabolite to parent drug concentration ratios in hair for the differentiation of tramadol intake from external contamination and passive exposure. *Forensic Sci Int*. 2012;223(1–3):330-334.
41. Tsanaclis L, Nutt J, Bagley K, Bevan S, Wicks J. Differentiation between consumption and external contamination when testing for cocaine and cannabis in hair samples. *Drug Test Anal*. 2014;6(Suppl 1(S1)):37-41.
42. Girod C, Staub C. Methadone and EDDP in hair from human subjects following a maintenance program: results of a pilot study. *Forensic Sci Int*. 2001;117(3):175-184.
43. Pragst F, Rothe M, Hunger J, Thor S. Structural and concentration effects on the deposition of tricyclic antidepressants in human hair. *Forensic Sci Int*. 1997;84(1–3):225-236.
44. Paterson S, Cordero R, McPhillips M, Carman S. Interindividual dose/concentration relationship for methadone in hair. *J Anal Toxicol*. 2003;27(1):20-23.
45. Kintz P, Bundeli P, Brenneisen R, Ludes B. Dose-concentration relationships in hair from subjects in a controlled heroin-maintenance program. *J Anal Toxicol*. 1998;22(3):231-236.
46. Gunther KN, Johansen SS, Nielsen MKK, Wicktor P, Banner J, Linnet K. Post-mortem quetiapine concentrations in hair segments of psychiatric patients - correlation between hair concentration, dose and concentration in blood. *Forensic Sci Int*. 2018;285:58-64.
47. Lees R, Kingston R, Williams TM, Henderson G, Lingford-Hughes A, Hickman M. Comparison of ethyl glucuronide in hair with self-reported alcohol consumption. *Alcohol Alcohol*. 2012;47(3):267-272.

48. Kronstrand R, Brinkhagen L, Nystrom FH. Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months. *Forensic Sci Int*. 2012;215(1–3):51-55.

49. Crunelle CL, Yegles M, De Doncker M, et al. Hair ethyl glucuronide concentrations in teetotalers: should we re-evaluate the lower cut-off? *Forensic Sci Int*. 2017;274:107-108.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Meier U, Colledge F, Imfeld S, et al. Distribution pattern of common drugs of abuse, ethyl glucuronide, and benzodiazepines in hair across the scalp. *Drug Test Anal*. 2019;1–20. https://doi.org/10.1002/dta.2679

3.4. Project 4: Incorporation of Forensically Relevant Substances into Hair via Exposure to Spiked Artificial Sweat Solutions

About Project 4

The aim of this project is to test the plausibility of the hypothesis set up in project 3 that the distribution patterns are caused by different sweat rates across the head. For the sweat rates to influence the concentrations found in hair samples, substances must be able to enter the hair via the sweat. This is being examined in this project. The project is currently underway, and no definite results are available yet. The project is presented in detail in the following. The results will be submitted to Drug Testing and Analysis.

Authors: Ulf Meier, Eva Scheurer, Franz Dussy

Contributions of Ulf Meier:

- Planning the study
- Doing the lab work
- Evaluating the results
- Writing the article

Status: Experiments in progress

3.4.1. Motivation for Project 4

In project 3, large concentration differences depending on sampling location on the head were identified. The sweating rates across the head showed a similar distribution as the concentrations. For sweat to cause the observed distribution patterns, substances must be able to enter the sweat from the bloodstream and be able to enter the hair from the sweat. The ability of substances to enter the hair via diffusion from the sweat is investigated in this project.

A number of studies have investigated the incorporation of substances into the hair from external sources in the context of contamination from e.g. cocaine powder.[11,77,85-87,104,105] These studies suffer from several limitations if the results are to be applied to the research question of this project. In some studies (e.g. Stout et al. 2006 and Ropero-Miller et al. 2012), cocaine was applied to the hair as a powder. While care was taken to apply the powder in a uniform way (e.g. by applying a defined amount of cocaine to gloved hands and subsequently rubbing the hair in the hands for a defined time), the actual amount of cocaine applied to the hair is unknown. Afterwards, different solutions such as artificial sweat solutions were applied to the hair to allow diffusion of the applied cocaine powder into the hair. This is likely a realistic model of external contamination from powder, but not very realistic regarding incorporation from sweat of a cocaine consuming person. In other studies, such as the studies of Schaffer et al. 2005 and Gerace et al. 2017, hair was subjected to cocaine solutions for prolonged periods. For example, Schaffer et al. soaked hair in a solution containing 1, 10, or 50 ng/µL cocaine for one hour,[86] and Cairns et al. 2004 also soaked hair in a 1 ng/µL solution for one hour.[85] For a model of incorporation via sweat, this is not a realistic model. Sweat on the hair dries fairly quickly and based on what little information is available from sweat patch experiments,[47,50,52,53,55,58] depending on the consumed amount, concentrations are likely lower than in these soaking experiments. Additionally, while the hair will be wet for only a short period of time, a regular cocaine consumer will expose their hair to cocaine containing sweat many times. Finally, the described studies investigated only cocaine or cocaine metabolites and only few studies investigate the incorporation of other substances such as opioids/opiates into the hair. Therefore, there is a need for a study investigating the extent of incorporation into hair from sweat with a realistic model of exposure and for an array of forensically interesting substances.

This study attempts to manage the mentioned limitations of previous studies by using a more realistic contamination model regarding concentrations, exposure style, exposure duration, exposure frequency, and including normal hygienic routine steps. The aim of this study is to test the plausibility of sweat rate differences across the head being the cause of the concentration differences that were observed across the head in the previous studies and further to test whether differing ability of the substances to enter the hair can explain the differences in the extent of the observed distribution patterns. This study is currently still underway. As such, the number of participants, exact methodology, interpretations, etc. are all subject to change before publication of the study. Some preliminary results are presented at the end of this section.

3.4.2. Materials and Methods

3.4.2.1. Study Design

Hair was collected from alcohol, DoA, and BZD-Z non-consuming acquaintances or employees of our institute after getting their hair cut. As such, the distal end of the hair was collected. The single hairs were manually formed into hair locks. One side of the manufactured hair locks was glued together using hot glue to fix the lock and prevent hair loss during soaking and cleaning steps. Hair locks were cut to a length of 3.5 cm from the glued segment. Hair locks were made so that before gluing the weight of the hair was approximately 40 mg.

Hair locks will be subjected to varying numbers of exposures to contaminated sweat solutions, cleaning steps, and exposures to a clean sweat solution. This is done to mimic a single consumption of substances or regular consumption of substances for different time periods. Hair samples will be soaked in sweat solutions with concentration levels low (EtG 0.05 ng/µl, DoA/BZD-Z 0.01 ng/µl), mid (EtG 0.1 ng/µl, DoA/BZD-Z 0.1 ng/µl), or high (EtG 0.2 ng/µl, DoA/BZD-Z 1.0 ng/µl). Hair soaking will be done as follows: A hair strand is placed into a 4 ml vial containing the spiked sweat solution for 10 seconds while gently moving the hair strand to ensure full wetting. The lock is removed from the bath while gently pulling along the edge of the vial to remove excess solution. The lock is placed on a paper towel and allowed to fully dry at room temperature as determined by inspection before repeating the soaking process. After every second soak, the hair lock is washed to simulate normal hair hygiene. Hair washing is done by rinsing the hair under running water for 20 seconds. Afterwards, 50 µl of shampoo is applied to the hair, rubbed in, and allowed to act on the hair for 1 minute. The hair is rinsed under running water for 30 seconds, placed on a paper towel, and allowed to dry. After complete drying, 30 µl of an artificial sebum-sweat emulsion is applied to the hair to restore the removed fat. This soaking-cleaning process is repeated until hair locks have been soaked for 1 time, 7 times, 28 times, or 56 times. For the 1-time and 7-times treatments, an extra lock is treated for each concentration level. For the extra lock, soaking and washing steps will be continued using a “clean” sweat solution containing no EtG, DoA, or BZD-Z until 28 soaks have been done. This is done to simulate a single or short-term consumption period, followed by a period of abstinence. After the hair has been soaked for their respective times, the glued section of the hair will be cut off and discarded.

3.4.2.2. Materials and Instrumentation

For all reference materials, internal standards, solvents, and instrumentation see sections 3.2 and 3.3. The artificial eccrine perspiration (pH 4.5, stabilized) and the artificial eccrine sweat-sebum emulsion (pH 4.5, not stabilized) were purchased from Pickering Laboratories Inc. (Mountain View, CA, USA).

3.4.2.3. Sample Preparation and LC-MS/MS Analysis

The locks will be washed for 3 minutes with 4 ml water and twice with 4 ml acetone. Each wash solution will be individually collected for analysis. Hair locks will be prepared and analyzed according to the combined sample preparation method (see sections 3.2 and 3.3). For all validation parameters see sections 3.2 and 3.3. Wash solutions will be analyzed as follows: The wash solutions will be spiked with internal standard mix. The acetone washes will be dried under nitrogen and resuspended in 1.5 mL water:MeOH 1:1. The water wash solutions and the resuspended acetone wash solutions will be added to an Oasis Max SPE column and prepared and analyzed with the combined sample preparation method. The concentrations in the wash solutions are normalized to the amount of washed hair. The concentration of the wash solutions is normalized to the hair amount as pg $_{\text{substance}}$/mg $_{\text{washed hair}}$.

3.4.3. Pre-experiment and Preliminary Results

As this is still work in progress no definite results can be presented yet. A pre-experiment has been conducted to test if the practical handling works and if sensible concentration levels were chosen. The pre-experiment will be briefly presented here. The three concentration levels low (EtG 0.01 ng/µl, DoA/BZD-Z 0.01 ng/µl), mid (EtG 0.1 ng/µl, DoA/BZD-Z 0.1 ng/µl), and high (EtG 0.2 ng/µl, DoA/BZD-Z 1.0 ng/µl) were chosen. Locks were soaked in the respective solution for 10 seconds. Afterwards, the hair locks were allowed to dry on a paper towel. After two rounds of soaking, the locks were washed as described above including the shampooing, but not including the application of the sebum-sweat emulsion. Locks were soaked in the spiked sweat solutions a total of 10 times. After the final soak the locks were washed with shampoo. The hair samples were then prepared and analyzed as described above, including analyzing the wash solutions. The experiment was conducted in triplicate. The results of the pre-experiment are presented in Table 1 and Table 2. The analysis of the wash solutions has not been validated. Values for the wash solutions are reported for samples with S/N ratios ≥ 10.

The substance concentrations in the hair locks seem to increase proportionally to the concentration in the sweat. For EtG, the low concentration did not yield measurable results. Therefore, the EtG concentration in the low level is increased 5-fold from 0.01 ng/µl in the pre-experiment to 0.05 ng/µl in the experimental plan. For the DoA, the low concentration yielded results that were generally below the LOQ/LOD, the mid concentration yielded results generally below the proposed abstinence cut-off for many of the substances by the SoHT of 200 pg/mg (range 24 pg/mg [BE] up to 213 pg/mg [MDA]), and the high concentration yielded results up to around 1500 pg/mg (range: 227 pg/mg [BE] to 1575 pg/mg [MDA]). The concentrations of the BZD-Z were generally higher than for EtG or DoA and ranged from non-detectable (zopiclone) to 35 pg/mg (oxazepam/bromazepam) for the low concentration, from 38 pg/mg (zopiclone) to 360 pg/mg (oxazepam) for the mid concentration, and from 420 pg/mg (zopiclone) to 3660 pg/mg (diazepam) for the high concentration level. The concentrations were quite substance dependent. EtG clearly showed the lowest incorporation. Of the DoA, benzoylecgonine showed the lowest incorporation while methadone on average showed the highest. This may be because benzoylecgonine is amphoteric in contrast to the

otherwise basic DoA. The BZD-Z generally showed a higher degree of incorporation than the DoA or EtG. Among the BZD-Z, zopiclone showed a much lower incorporation than the other substances, which may be due to its weak basicity compared to the other BZD-Z. The second acetone wash solution typically contained only relatively low concentrations of EtG and DoA, no higher than 15 % of the concentration in the hair, except for MTD and EDDP. This is in contrast with the BZD-Z, for which the second acetone washing step still contained about 30 % to 60 % of the concentrations found in the hair. The applied washing procedure was chosen as it follows the harmonized hair washing/decontamination procedure currently being discussed in Switzerland.

The results show that after soaking hair locks ten times in spiked sweat solutions, relevant concentrations of EtG, DoA, and BZD-Z were found in or on the surface of the hair and the "contamination" could not be fully removed by our washing procedure. These preliminary results at least do not contradict our working hypothesis that the distribution patterns observed in projects 1 and 3 are caused by differences in sweating rates. However, they do not explain the observed extent of the distribution patterns. For example, cocaine generally showed high differences across the head while methadone showed low differences. The preliminary results show that methadone seems to be incorporated better into the hair via sweat than cocaine. This is the opposite behavior than what would be expected if the ability of substances to enter the hair via the sweat is the determining factor for the extent of the distribution patterns across the head. This suggests that the extent of the distribution patterns is not determined by the propensity of substances to enter the hair from the sweat and is more likely determined by the ability of the substances to enter the sweat from the blood. The results will also be relevant for routine forensic hair interpretation. From the preliminary results it is likely that they will show that all tested substances have the ability to enter the hair and can be incorporated strongly enough to not be removed by the applied standard washing procedure. This is problematic for interpretation of results as it opens up the possibility of transfer of substances of a consumer to a non-consumer via sweat, including transfer of drug metabolites.
As these results are only preliminary they should be interpreted cautiously and a more thorough discussion will be held when definite results are available. Additionally, no reliable data on the concentrations of the substances in the sweat in relationship to blood concentrations are available. This should be the focus of future studies.

Table 1 EtG and DoA concentrations in hair locks soaked 10 times in sweat solutions spiked with low, mid or high concentrations. Normalized concentrations of EtG and DoA in the water, first acetone, and second acetone wash solutions. (*) Approximate value as below LOQ. Concentrations are given as pg/mg.

Sample	EtG	Coc	BE	NorCoc	CE	Mor	MAM	Codeine	AcCo	MTD	EDDP	Amph	Meth	MDMA	MDA	Ritalin
Hair-Low-1	-	-	-	11	19	-	-	6*	9	19*	15	11	8*	10*	-	-
Hair-Low-2	-	-	-	10	14	-	-	-	8	19*	14	18	5*	16*	-	-
Hair-Low-3	-	-	-	8	14	-	-	5*	6	13*	11	9	6*	10*	-	-
Hair-Mid-1	7.8	76	28	121	107	66	70	55	97	204	142	90	71	113	121	61
Hair-Mid-2	8.7	85	32	126	110	63	62	49	89	178	114	165	127	189	213	77
Hair-Mid-3	8.1	55	24	78	67	37	39	33	52	116	73	88	68	104	113	46
Hair-High-1	16.8	620	298	933	746	512	545	420	694	1460	1002	865	679	1065	1027	510
Hair-High-2	16.7	652	282	1057	771	514	469	395	650	1463	928	1328	1053	1517	1575	572
Hair-High-3	16.4	490	227	802	604	435	425	340	577	1192	771	854	679	965	1052	439
H_2O-Low-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2O-Low-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2O-Low-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2O-Mid-1	5.6	-	-	23	23	20	19	-	22	19	19	22	18	22	22	15
H_2O-Mid-2	2.3	-	-	14	16	11	-	-	-	15	14	22	18	18	28	9
H_2O-Mid-3	2.1	-	-	12	12	8	8	-	-	13	11	18	10	15	24	8
H_2O-High-1	3.9	66	34	89	73	64	63	67	70	86	79	111	84	104	116	48
H_2O-High-2	6.0	117	57	152	127	101	108	108	140	143	142	258	212	199	240	101
H_2O-High-3	4.2	86	37	116	92	80	76	70	88	122	105	167	147	157	167	67
Ac-1-Low-1	-	6	-	-	5	-	-	-	-	5	-	-	-	-	-	-
Ac-1-Low-2	-	8	-	-	5	-	-	-	-	-	-	-	-	-	-	-
Ac-1-Low-3	-	8	-	-	5	-	-	-	-	-	-	-	-	-	-	-
Ac-1-Mid-1	-	18	-	13	19	9	10	9	12	39	24	10	8	11	11	9
Ac-1-Mid-2	-	21	-	12	19	7	7	6	11	39	22	18	13	15	19	7
Ac-1-Mid-3	-	14	-	8	13	-	-	-	7	29	17	9	7	9	10	-
Ac-1-High-1	2.2	130	42	144	155	89	91	75	128	401	232	153	134	178	166	98
Ac-1-High-2	-	115	26	123	145	62	64	53	106	438	227	201	162	186	210	73
Ac-1-High-3	-	91	21	110	122	53	58	53	90	394	205	130	112	139	145	62
Ac-2-Low-1	-	6	-	-	-	-	-	-	-	6	-	-	-	-	-	-
Ac-2-Low-2	-	7	-	-	-	-	-	-	-	3	-	-	-	-	-	-
Ac-2-Low-3	-	8	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Ac-2-Mid-1	-	10	-	3	10	-	-	-	-	20	11	-	-	-	-	-
Ac-2-Mid-2	-	20	-	5	18	-	-	-	7	32	18	-	-	-	-	-
Ac-2-Mid-3	-	12	-	3	11	-	-	-	-	18	10	-	-	-	-	-
Ac-2-High-1	-	45	-	28	63	5	11	-	32	191	98	11	9	13	14	16
Ac-2-High-2	-	74	-	40	99	5	12	-	46	284	137	15	11	19	19	20
Ac-2-High-3	-	62	-	43	92	5	17	9	47	266	136	14	11	19	19	23

Abbreviations: EtG (Ethyl glucuronide); Coc (Cocaine); BE (Benzoylecgonine); NorCoc (Norcocaine); CE (Cocaethylene); Mor (Morphine); MAM (6-Monoacetylmorphine); AcCo (Acetylcodeine); MTD (Methadone); EDDP (2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine); Amph (Amphetamine); Meth (Methamphetamine); MDMA (3,4-Methylenedioxy-N-methylamphetamine); MDA (3,4-Methylenedioxyamphetamine); Ritalin (Methylphenidate)

Table 2 Concentration of BZD-Z in hair locks soaked 10 times in sweat solutions spiked with low, mid or high concentrations. Normalized concentrations of BZD-Z in the water, first acetone, and second acetone wash solutions. (*) Approximate value as below LOQ. Concentrations are given as pg/mg.

Sample	Di	Nor	Tem	Ox	Lor	Brom	Mid	Hy-Mid	Clob	Clon	Am-Clon	Lormet	Triaz	Fluni	7-AFN	Alpr	Desflur	Zolpidem	Zopiclone
Hair-Low-1	24	23	19	35	23	35	30	25	14	20	9.9*	27	23	17	12	14	22	15	6.9*
Hair-Low-2	23	23	19	32	21	33	28	24	14	20	9.7*	26	23	16	11	14	23	14	6.2*
Hair-Low-3	23	20	15	27	18	16*	21	18	11	16	6.5*	20	17	14	8.0	10	19	9.7	-
Hair-Mid-1	296	281	220	360	271	335	317	284	161	242	204	267	154	306	141	161	257	162	72
Hair-Mid-2	351	282	238	336	223	200	319	263	185	216	232	228	141	296	122	139	250	146	63
Hair-Mid-3	266	207	160	219	150	150	213	171	124	152	156	158	88	207	83	93	182	87	38
Hair-High-1	2930	2561	1966	2902	2114	2474	2790	2405	1485	1984	1886	2154	1195	2623	1052	1318	2248	1265	582
Hair-High-2	3660	2974	2187	3209	2158	1968	2920	2506	1613	2156	2229	2155	1296	2846	1145	1287	2619	1212	489
Hair-High-3	2959	2465	1838	2819	1899	1785	2556	2174	1359	1870	1813	1908	1103	2495	1023	1102	2148	1050	420
H_2O-Low-1	2.2	2.1	-	-	2.1	-	-	-	2.2	2.7	-	2.8	-	2.3	1.7	-	-	1.9	-
H_2O-Low-2	2.7	2.9	-	-	2.3	-	-	-	1.9	2.4	-	-	-	2.0	1.6	-	-	1.7	-
H_2O-Low-3	1.6	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	0.9	-
H_2O-Mid-1	32	38	34	23	27	10	26	33	29	36	26	41	42	32	28	28	35	29	18
H_2O-Mid-2	25	30	23	16	21	-	18	24	21	22	19	28	27	23	19	16	23	16	8.5
H_2O-Mid-3	22	25	25	14	17	-	16	22	16	24	17	25	23	20	18	18	22	14	8.5
H_2O-High-1	186	232	183	130	163	20	138	181	136	202	161	221	198	164	155	125	204	106	59
H_2O-High-2	250	302	266	164	221	30	183	254	198	270	213	295	307	232	210	216	276	165	97
H_2O-High-3	218	270	217	157	206	52	195	234	158	230	172	256	240	181	163	155	232	125	70
Ac-1-Low-1	6.2	6.2	4.8	5.0	4.6	2.9	7.5	6.0	3.9	5.2	-	6.6	6.4	5.1	1.8	3.7	5.7	3.1	1.6
Ac-1-Low-2	5.1	5.0	4.2	3.3	3.9	2.0	5.3	4.5	3.3	4.4	-	5.4	4.8	4.0	1.2	2.8	4.7	2.4	1.1
Ac-1-Low-3	4.1	4.1	3.2	2.9	2.4	1.7	5.0	3.1	2.3	2.9	-	3.9	4.0	3.0	-	2.3	3.3	1.5	0.8
Ac-1-Mid-1	47	48	39	31	34	23	59	44	29	42	6.5	52	46	37	8.5	26	43	24	12
Ac-1-Mid-2	54	53	43	29	34	15	63	44	35	44	6.7	57	50	43	9.1	28	46	21	8.9
Ac-1-Mid-3	44	41	31	20	24	12	48	31	24	31	4.1	43	35	30	5.4	20	38	15	6.3
Ac-1-High-1	534	577	433	388	400	180	623	497	320	479	98	613	524	411	117	298	509	231	108
Ac-1-High-2	705	727	524	366	435	222	778	583	369	532	89	755	612	478	107	329	622	229	93
Ac-1-High-3	653	670	482	431	460	245	756	574	343	556	109	695	564	455	131	297	601	212	82
Ac-2-Low-1	8.9	8.4	6.0	4.9	5.4	3.6	9.8	7.4	4.8	7.1	-	9.1	7.4	6.1	1.0	4.2	7.8	3.0	-
Ac-2-Low-2	6.4	6.3	4.5	3.2	3.7	2.2	6.6	4.7	3.2	5.3	-	6.8	4.8	4.3	0.6	2.4	5.5	1.9	-
Ac-2-Low-3	2.9	3.1	2.2	-	1.6	1.3	3.6	2.4	1.5	2.0	-	2.8	2.2	1.8	-	-	2.3	1.0	-
Ac-2-Mid-1	37	34	23	17	19	18	37	25	18	26	-	34	28	23	2.8	15	30	11	4.3
Ac-2-Mid-2	50	48	38	27	29	17	62	42	29	35	-	48	43	35	4.7	24	42	20	6.9
Ac-2-Mid-3	33	32	22	16	16	9.6	33	23	16	21	-	30	24	19	2.2	12	28	9.9	4.3
Ac-2-High-1	377	357	243	183	201	142	370	276	175	251	27	322	265	227	34	146	313	101	38
Ac-2-High-2	469	480	323	236	256	173	525	404	232	319	34	444	357	295	45	193	405	144	53
Ac-2-High-3	517	498	324	242	281	176	540	408	219	348	39	444	353	301	50	194	426	150	58

Abbreviations: Di (Diazepam); Nor (Nordazepam); Tem (Temazepam); Ox (Oxazepam); Lor (Lorazepam); Brom (Bromazepam); Mid (Midazolam); Hy-Mid (Alpha-Hydroxymidazolam); Clob (Clobazam); Clon (Clonazepam); Am-Clon (7-Aminoclonazepam); Lormet (Lormetazepam); Triaz (Triazolam); Fluni (Flunitrazepam); 7-AFN (7-Aminoflunitrazepam); Alpr (Alprazolam); Desflur (N-Desalkylflurazepam);

3.5. Project Kratom: Development, Validation, and Application of an LC-MS/MS Method for Mitragynine and 7-Hydroxymitragynine Analysis in Hair

About Project Kratom

To the best of our knowledge, this manuscript describes the first published hair analysis method for mitragynine and 7-Hy-Mitra. A method for mitragynine and 7-Hy-Mitra analysis was developed, fully validated, and applied to the entire scalp hair of a self-reported consumer of 3 g of Kratom daily to describe the concentration distribution. Additionally, the method was applied to 300 routine samples to investigate the prevalence of Kratom consumption in our population. The article will be submitted to Drug Testing and Analysis as a short communication.

Authors: Ulf Meier, Katja Mercer-Chalmers-Bender, Eva Scheurer, Franz Dussy

Contributions of Ulf Meier:

- Sample collection
- Development of analytical method
- Sample preparation, analysis, and evaluation
- Writing the short communication

CRediT Statement:

Ulf Meier:	Conceptualization, formal analysis, investigation, visualization, methodology, project administration, writing – original draft
Katja Bender:	Writing – review & editing
Eva Scheurer:	Supervision, writing – review & editing
Franz Dussy:	Conceptualization, supervision, writing – review & editing

Status: Published

Received: 5 November 2019 | Revised: 4 December 2019 | Accepted: 5 December 2019

DOI: 10.1002/dta.2746

WILEY

SHORT COMMUNICATION

Development, validation, and application of an LC–MS/MS method for mitragynine and 7-hydroxymitragynine analysis in hair

Ulf Meier | Katja Mercer-Chalmers-Bender | Eva Scheurer | Franz Dussy

Department of Biomedical Engineering, University of Basel Institute of Forensic Medicine, Switzerland

Correspondence

Ulf Meier, Department of Biomedical Engineering, University of Basel Institute of Forensic Medicine, Switzerland.
Email: ulf.meier@bs.ch

Abstract

The entire scalp hair of a self-declared Kratom consumer of 3 grams per day was acquired during an ethical committee approved study. As no values of the concentration in hair of the two Kratom alkaloids mitragynine or 7-hydroxymitragynine were found in the literature, an already established method for the analysis of benzodiazepines/z-substances was extended for the detection of mitragynine and 7-hydroxymitragynine with LC–MS/MS, and successfully validated. The limits of detection and quantification for mitragynine were 2 pg/mg and 4 pg/mg, respectively. Those of 7-hydroxymitragynine were 20 pg/mg and 30 pg/mg, respectively. The method was applied to the entire scalp hair, divided in 91 regions, of the study participant. A narrow mitragynine concentration distribution with values between 1054 pg/mg and 2244 ng/mg (mean 1517 ng/mg) and no clear scalp region associated distribution pattern was obtained. 7-Hydroxymitragynine was not detected in any hair sample. After validation, the method was established as routine and subsequently 300 samples (mainly abstinence controls for drugs of abuse) were analyzed, allowing the investigation of the prevalence of Kratom consumption in our population. None of the analyzed routine hair samples were positive for mitragynine or 7-hydroxymitragynine, providing no evidence that Kratom consumption is prevalent in the investigated population.

KEYWORDS

distribution pattern, hair analysis, kratom mitragynine, LC–MS/MS

1 | INTRODUCTION

Mitragyna speciosa also known as Kratom, Ketum, or Biak-Biak[1] is an evergreen tree that is native to Southeast Asia.[2] Kratom has been used for many centuries for its stimulating and opioid-like effects, such as for facilitating hard labor or recreational use.[3] Kratom is typically consumed by chewing the leaves, drinking a tea brewed from the leaves, or by swallowing powdered leaves.[4] No exact data on prevalence of Kratom consumption are available. From online surveys and the availability in shops, it seems that Kratom consumption has seen an increase in many European countries and in the United States.[3,5] It is used as an opioid substitute in Southeast Asia as well as, for example, in the USA.[3,4,6,7] The legal status of Kratom is diverging within Europe. It is a controlled substance in Switzerland,[8] Denmark, and Poland[9] but remains legal in many European countries.

The pharmacology of Kratom is poorly understood. In low doses it produces stimulating effects and in high doses opioid-like effects. Some effects of Kratom include nausea, euphoria, constipation, weight loss, appetite suppression, and antinociception.[1,3,10-12] Mitragynine and 7-hydroxymitragynine (7-Hy-Mitra) are considered to be the most important psychoactive components, but there are many other alkaloids that are suspected of influencing the

psychoactive effects of Kratom. The structures of both compounds are presented in Figure 1. They are both selective μ-opioid receptor partial agonists.[5,10,13,14] Mitragynine is the most abundant alkaloid of Kratom with about 66% of the total alkaloid content, while 7-Hy-Mitra is present at lower concentrations of about 2% of the total alkaloids.[15] The concentrations have been reported to be strongly dependent on the strain and origin of the Kratom.[16] It is possible that 7-Hy-Mitra is a metabolite of mitragynine but there are contradictory reports.[5,14]

During a recently published study,[17] the entire scalp hair of a self-reported cocaine and Kratom consumer was collected. The participant reported regular consumption of a moderate amount of ca. 3 g/day.[6]

A literature search yielded no published analytical methods or reference values for mitragynine or 7-Hy-Mitra in hair. Therefore, an existing LC–MS/MS method for the analysis of benzodiazepines/z-substances (BZD-Z) was extended for the detection of mitragynine and 7-Hy-Mitra and fully validated. These alkaloids were chosen as they are considered the most important psychoactive compounds in Kratom. The method was applied to gain insight into the concentration distribution across the scalp, as the sampling site could have a strong impact on the test results as previously demonstrated (Meier et al., 2019).[17] Additionally, the method was established as a routine analysis method, which allowed the study of the prevalence in our routine population.

2 | MATERIALS AND METHODS

2.1 | Sampling

The entire head hair of the study participant was collected in a raster with sample areas of around 3 × 3 cm, yielding a total of 91 samples. The corner points of each sample area were marked, and photographs were taken after hair collection to determine the exact sampling location on the scalp. Each hair lock was shortened to a uniform length of 3 cm to allow comparison of all samples.

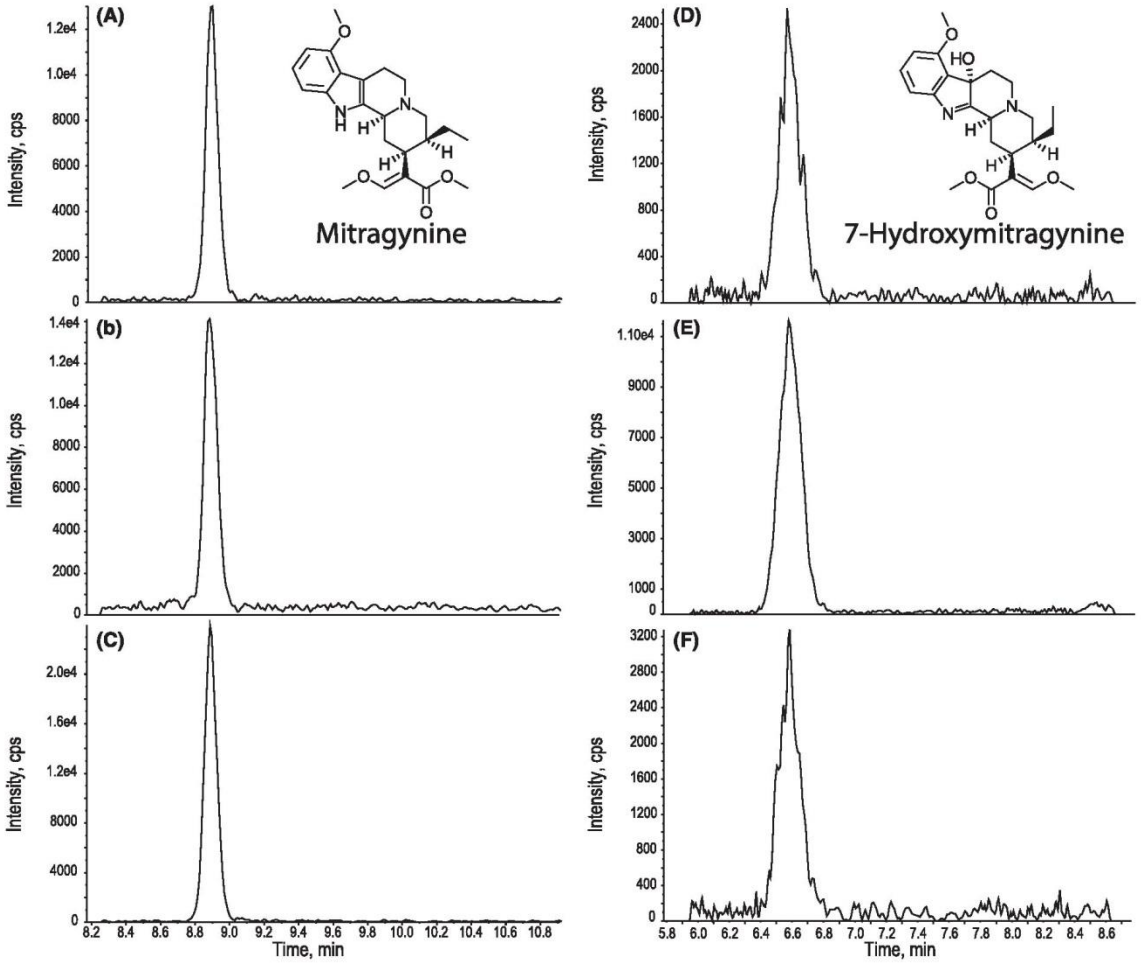


FIGURE 1 Chromatograms of each mass transition of mitragynine (A: 399.1/238.1; B: 399.1/226.1; C: 399.1/174.0); and 7-Hy-Mitra (D: 415.2/238.1; E: 415.2/226.2; F: 415.2/190.0) of a blank matrix sample spiked with the concentration at the LOQ (mitragynine 4 pg/mg, 7-Hy-Mitra 30 pg/mg). The structures of mitragynine and 7-Hy-Mitra are presented

2.2 | Routine hair samples

After validation, the method was established as routine, and applied to all hair samples that were analyzed for drugs of abuse (DoA). This allowed us to determine whether Kratom use is prevalent in our routine population. Hair samples were mostly collected in the context of abstinence controls for driving license regranting procedures. The method was applied to hair samples of 300 persons. Applying the cut-offs recommended by the Society of Hair Testing the following number of hair samples tested positive for DoA: cocaine 55, opiates 10, methadone 14, amphetamines 19, methylphenidate 9 (cut-off 10 pg/mg).

2.3 | Chemicals

Mitragynine, 7-Hy-Mitra, and mitragynine-D3 were supplied by Cerilliant (Round Rock, Texas). Stock solutions containing 10 ng/µL in methanol were prepared. Working standard solutions with various concentrations were prepared from stock solutions as needed. All solutions were stored at 4°C. The LC–MS solvents water, acetonitrile, and methanol were obtained in analytical grade purity from Machery-Nagel AG (Oensingen, Switzerland). Formic acid puriss p.a. (98%), ammonium formate (≥ 99.0%), 2-propanol (≥ 99.5%), and acetone (≥ 99.5%) were purchased from Roth (Arlesheim, Switzerland). The deionized water used for washing the hair was produced in-house.

2.4 | Sample preparation

Sample preparation was done using a published method for the combined sample preparation of EtG, DoA, and BZD-Z.[17,18] Hair strands were briefly (1–2 min) washed with 3 mL each of water, 2-propanol, and acetone. Approximately 20 mg hair was pulverized in a 2 mL Eppendorf tube with a ball mill (MM 200 ball mill, Retsch, Haan, Germany). To the powdered hair, 10 ng (10 µL, 1 ng/µL) mitragynine-D3 was added. The hair was extracted for 4 h with 1.5 mL water:methanol 1:1 on an overhead shaker. The sample was centrifuged at 13000 rpm (13793 × g) for 10 minutes and the supernatant loaded onto an Oasis Max SPE cartridge. The fraction of the supernatant passing through the SPE cartridge was collected. The cartridge was washed with 2 mL MeOH and the wash solution was combined with the first fraction. The combined solution was dried under a nitrogen flow and reconstituted in 0.5 mL ammonium formate buffer (200 nM, pH 5).

2.5 | LC-MRM analysis

The method was integrated into a published method for the analysis of BZD-Z.[17] Analysis was done using an Ultimate 3000 high performance liquid chromatograph (Thermo Fisher Scientific, Reinach, Switzerland) coupled to a 5500 QTrap triple quadrupole mass spectrometer (Sciex, Brugg, Switzerland).

A Phenomenex Kinetex 50 × 2.1 mm 2.6 µm column (Brechbühler, Schlieren, Switzerland), with a flow rate of 500 µL/min was used with the following LC program: ammonium formate buffer (10 mM ammonium formate set to pH 3.4 with formic acid) was used for the gradient program with 90% (2 min hold) to 40% at 12 min and 5% at 13 min adjusted to 100% with methanol. An injection volume of 20 µL was used. The instrument was operated in positive APCI mode with a probe temperature (TEM) of 550°C, curtain gas (CUR) of 30 psi, a nebulizer gas (GS1) of 45 psi, a nebulizer current (NC) of 3 V, and an ionspray voltage (IS) of 5500 V. The entrance potential (EP) and collision cell exit potential (CXP) were set to 10 V and 42 V, respectively. The collision gas (CAD) was set to 8. The substance specific instrument parameters for mitragynine, 7-Hy-Mitra, and mitragynine-D3 are shown in Table 1. Both mitragynine and 7-Hy-Mitra concentrations were calculated with mitragynine-D3 as an internal standard and using the mean of all transitions as this was determined to give the best accuracy and precision. Data acquisition was done using a scheduled MRM program with a target cycle time of 0.3 s.

2.6 | Validation

Validation was done according to the guidelines of the German Society of Forensic Toxicology and Chemistry (GTFCh).[19] The validation parameters selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), bias, precision, accuracy, extraction efficiency, and matrix effects were determined.

Selectivity was tested by measuring six and eight blank samples from different individuals with addition and without addition of the internal standard, respectively. Linearity was determined by six replicate measurements of matrix calibration rows with levels 5, 50, 500, 1250, 2500, 3750, and 5000 pg/mg for mitragynine and 50, 500, 1250, 2500, 3750, and 5000 pg/mg for 7-Hy-Mitra. Grubb's test was used to identify outliers. Mandel's test was used to check for non-linearity. An F-test on the lowest and highest calibration levels was used to test for homoscedasticity. The LOD and LOQ were determined from the signal-to-noise ratio (s/n) of samples spiked at low concentrations. Accuracy, bias, inter-day, and intra-day precision were determined by spiking blank matrix in duplicate with three concentration levels (low: 50 pg/mg, mid: 500 pg/mg, high: 5000 pg/mg) on 8 consecutive days as described by the GTFCh. Recovery and matrix effects were determined for two concentration levels (low: 50 pg/mg, high: 5000 pg/mg). For this, blank hair extracts from five different sources were prepared and standards were spiked to the dried SPE extracts (A). The spiked extracts (A) were compared with standards prepared in buffer solution (B). The matrix effect was calculated as A/B*100. Five blank hair samples were spiked with standards and prepared according to the method (C). The recovery was determined by comparing the signal intensity of the spiked extracts with spiked blank hair samples (recovery = C/A*100).

TABLE 1 Substance specific instrument parameters for mitragynine, 7-Hy-Mitra, and mitragynine-D3; mass transitions (Q1→Q3), expected retention time (RT), and collision energy (CE)

	Q1 → Q3 (m/z)	RT (min)	CE (V)
Mitragynine 1/2/3	399.2 → 238.1/226.1/174.0	8.92	35/34/44
7-Hy-Mitra 1/2/3	415.2 → 238.1/226.2/190.0	6.52	35/34/44
Mitragynine-D3 1/2/3	402.2 → 238.1/226.1/177.0	8.92	34/32/37

3 | RESULTS AND DISCUSSION

3.1 | Validation

The method was successfully validated for mitragynine and 7-Hy-Mitra. Validation parameters are presented in Table 2. No interfering peaks were detected in the blank samples. Linearity was given over the whole range with no detected outliers and linearity was confirmed by Mandel's test. Homoscedasticity was not given for mitragynine or 7-Hy-Mitra over the calibration range. This was expected, given the large calibration range and a weighting factor of 1/x was applied. As accuracy and precision values were acceptable for low, mid, and high concentrations, this was considered acceptable. The LOD of mitragynine and 7-Hy-Mitra were determined from the spiking experiments as 2 pg/mg and 20 pg/mg, respectively. The LOQ of mitragynine and 7-Hy-Mitra were determined as 4 pg/mg and 30 pg/mg, respectively. Chromatograms of all mitragynine and 7-Hy-Mitra mass transitions at the LOQ are presented in Figure 1. The instrument parameters of the ion source could not be optimized for the analysis of mitragynine or 7-Hy-Mitra as the analytes were integrated into an existing method. It is possible that optimizing the ion source parameters could increase the sensitivity.

3.2 | Chronic consumer and routine samples

Mitragynine was detected in every hair sample of the participant with concentrations between 1054 pg/mg and 2244 pg/mg (mean 1517 pg/mg, median 1487 pg/mg, standard deviation 240 pg/mg). The distribution pattern is shown as a projection onto the participant's head in Figure 2. The mitragynine concentration of every sample area is shown in Figure 3. The distribution showed a narrow concentration range with no clear distribution pattern. The results contrast with the distribution found by Meier et al. for most DoA, as most show higher

TABLE 2 Validation parameters accuracy bias, precision, matrix effect, and recovery for mitragynine and 7-Hy-Mitra. The intra-day precision was equal to the inter-day precision in all cases

	Accuracy bias (%)			Precision (%)			Matrix effect (%)		Recovery (%)]	
	Low	Mid	High	Low	Mid	High	Low	High	Low	High
Mitragynine	−1.1	−0.5	−1.6	9.4	4.7	1.6	115 ± 5	83 ± 3	87 ± 5	95 ± 3
7-Hy-Mitra	−5.5	0.2	0.3	9.8	6.8	6.9	105 ± 7	91 ± 3	80 ± 5	79 ± 4

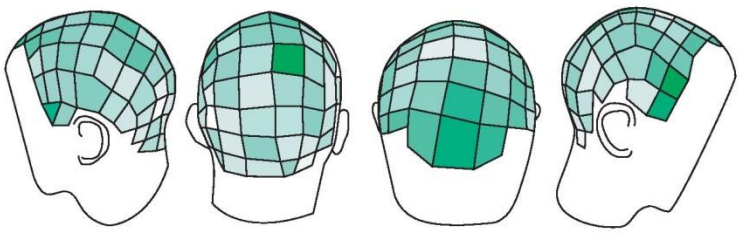


FIGURE 2 Distribution pattern of mitragynine of the self-reported Kratom consumer. Darker colors show areas with higher concentrations

FIGURE 3 Mitragynine concentrations in pg/mg of the hair sample areas of the self-reported Kratom consumer. The graph must be read as: top, forehead; left, left temple; right, right temple; bottom, neck

concentrations on the periphery of the hairline.[17] 7-Hy-Mitra was not detected in any of the hair samples, likely due to the lower abundance of 7-Hy-Mitra in the plant material compared with mitragynine, in combination with the higher LOD of 7-Hy-Mitra. The 7-Hy-Mitra concentration of the Kratom consumed by the study participant is unfortunately unknown. The self-reported consumption amount of 3 g/day was considered reliable as the participant had no reason to lie, reported consuming it regularly every day, and provided exact information as to which internet site the Kratom was ordered from and the size and cost of the order.

Neither mitragynine nor 7-Hy-Mitra were detected in any of the 300 measured routine hair samples, showing that the prevalence of consumption in our routine population in Switzerland is likely low or possibly not existent and that there was not a shift to Kratom from the traditional DoA.

4 | CONCLUSION

A method for mitragynine and 7-Hy-Mitra quantification was developed and fully validated. The method was applied to the hair of a self-reported Kratom consumer and the distribution pattern of mitragynine concentrations was described. The analysis of 300 routine samples at our laboratory yielded no positive results, showing a low prevalence of Kratom consumption in our routine population. The investigated population is likely to poorly reflect the general population of Switzerland. Therefore, the results do not show that there is generally a low prevalence of Kratom consumption. Trends in Kratom consumption should be monitored as legislation continues to change.

ORCID

Ulf Meier https://orcid.org/0000-0002-8284-9467

Katja Mercer-Chalmers-Bender https://orcid.org/0000-0002-3843-9065

Eva Scheurer https://orcid.org/0000-0002-2742-0422

REFERENCES

1. Warner ML, Kaufman NC, Grundmann O. The pharmacology and toxicology of kratom: from traditional herb to drug of abuse. *Int J Leg Med*. 2016;130(1):127-138.
2. Trakulsrichai S, Sathirakul K, Auparakkitanon S, et al. Pharmacokinetics of mitragynine in man. *Drug Des Devel Ther*. 2015;9:2421-2429.
3. Henningfield JE, Fant RV, Wang DW. The abuse potential of kratom according the 8 factors of the controlled substances act: implications for regulation and research. *Psychopharmacology (Berl)*. 2018;235(2):573-589.
4. Singh D, Narayanan S, Vicknasingam B. Traditional and non-traditional uses of Mitragynine (Kratom): a survey of the literature. *Brain Res Bull*. 2016;126(Pt 1):41-46.
5. Kruegel AC, Uprety R, Grinnell SG, et al. 7-Hydroxymitragynine is an active metabolite of mitragynine and a key mediator of its analgesic effects. *ACS Cent Sci*. 2019;5(6):992-1001.
6. Grundmann O. Patterns of Kratom use and health impact in the US – results from an online survey. *Drug Alcohol Depend*. 2017;176:63-70.
7. Vicknasingam B, Narayanan S, Beng GT, Mansor SM. The informal use of ketum (*Mitragyna speciosa*) for opioid withdrawal in the northern states of peninsular Malaysia and implications for drug substitution therapy. *Int J Drug Policy*. 2010;21(4):283-288.
8. Eidgenössisches Departement des Innern. Betäubungsmittelverzeichnisverordnung. 2011; https://www.admin.ch/opc/de/classified-compilation/20101220/index.html. Accessed 9 April 2019.
9. European Monitoring Centre for Drugs and Drug Addiction. Kratom (*Mitragyna speciosa*) drug profile. http://www.emcdda.europa.eu/publications/drug-profiles/kratom. Accessed 9 April 2019.
10. Brown PN, Lund JA, Murch SJ. A botanical, phytochemical and ethnomedicinal review of the genus *Mitragyna korth*: implications for products sold as kratom. *J Ethnopharmacol*. 2017;202:302-325.
11. Matsumoto K, Horie S, Ishikawa H, et al. Antinociceptive effect of 7-hydroxymitragynine in mice: discovery of an orally active opioid analgesic from the Thai medicinal herb *Mitragyna speciosa*. *Life Sci*. 2004;74(17):2143-2155.
12. Matsumoto K, Mizowaki M, Suchitra T, et al. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic systems. *Eur J Pharmacol*. 1996;317(1):75-81.
13. Kruegel AC, Gassaway MM, Kapoor A, et al. Synthetic and receptor signaling explorations of the Mitragyna alkaloids: Mitragynine as an atypical molecular framework for opioid receptor modulators. *J Am Chem Soc*. 2016;138(21):6754-6764.
14. Manda VK, Avula B, Ali Z, Khan IA, Walker LA, Khan SI. Evaluation of in vitro absorption, distribution, metabolism, and excretion (ADME) properties of mitragynine, 7-hydroxymitragynine, and mitraphylline. *Planta Med*. 2014;80(7):568-576.
15. Ponglux D, Wongseripipatana S, Takayama H, et al. A new indole alkaloid, 7 alpha-Hydroxy-7H-mitragynine, from *Mitragyna speciosa* in Thailand. *Planta Med*. 1994;60(6):580-581.
16. Kikura-Hanajiri R, Kawamura M, Maruyama T, Kitajima M, Takayama H, Goda Y. Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant "kratom" (*Mitragyna speciosa*) by LC-ESI-MS. *Forensic Toxicol*. 2009;27(2):67-74.
17. Meier U, Colledge F, Imfeld S, et al. Distribution pattern of common drugs of abuse, ethyl glucuronide, and benzodiazepines in hair across the scalp. *Drug Test Anal*. 2019;11(10):1522-1541.
18. Meier U, Briellmann T, Scheurer E, Dussy F. Sample preparation method for the combined extraction of ethyl glucuronide and drugs of abuse in hair. *Drug Test Anal*. 2018;10(4):701-710.
19. Peters FT, Hartung M, Herbold M, Schmitt G, Daldrup T, Musshoff F. Anhang B zur Richtlinie der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen. Anforderungen and die Validierung von Analysemethoden. *Toxichem Krimtech*. 2009;76(3):185-208.

How to cite this article: Meier U, Mercer-Chalmers-Bender K, Scheurer E, Dussy F. Development, validation, and application of an LC–MS/MS method for mitragynine and 7-hydroxymitragynine analysis in hair. *Drug Test Anal*. 2020;1–5. https://doi.org/10.1002/dta.2746

3.6. Project CBD: Cannabinoid Concentrations in Blood and Urine after Smoking Cannabidiol Joints

About Project CBD

This publication describes a single person study investigating the possibility of reaching THC blood concentrations above the legal threshold for driving in Switzerland after smoking a single, legally available, cannabidiol joint. It is a one-participant study that is not part of the main work described in this thesis. However, there was an urgent need for some data as toxicologist in Switzerland are regularly confronted with CBD cannabis.

Authors: Ulf Meier, Franz Dussy, Eva Scheurer, Katja Mercer-Chalmers-Bender, Sarah Hangartner

Contributions of Ulf Meier:

- Involved in planning the study
- Interpreting data
- Writing the article

CRediT Statement:

Ulf Meier:	Conceptualization, investigation, writing – original draft
Franz Dussy:	Conceptualization, investigation, writing – review & editing
Eva Scheurer:	Writing – review & editing
Katja Bender:	Investigation, writing – review & editing
Sarah Hangartner:	Conceptualization, formal analysis, methodology, investigation, supervision, validation, writing – review & editing

Status: Published

Forensic Science International 291 (2018) 62–67

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Cannabinoid concentrations in blood and urine after smoking cannabidiol joints

Ulf Meier*, Franz Dussy, Eva Scheurer, Katja Mercer-Chalmers-Bender, Sarah Hangartner

Institute of Forensic Medicine of the University of Basel, Health Department Basel-Stadt, Pestalozzistrasse 22, CH-4056 Basel, Switzerland

ARTICLE INFO

Article history:
Received 18 May 2018
Received in revised form 3 August 2018
Accepted 6 August 2018
Available online 11 August 2018

Keywords:
Cannabidiol
Cannabinoids
Abstinence testing
Driving capacity
GC–MS/MS

ABSTRACT

In Switzerland, the sale of cannabis with tetrahydrocannabinol (THC) content less than 1% has recently been legalized. As a consequence, cannabis with low THC and high cannabidiol (CBD) values up to approximately 25% is legally available on the market. In this study, we investigated cannabinoid blood and urine concentrations of a naive user and of a modeled chronic user after smoking a single CBD joint. Chronic use was modeled as smoking 2 joints per day for 10 days. Joints contained 200 mg of cannabis with THC concentrations of 0.94% and 0.8% and CBD concentrations of 23.5% and 17% in the naive-smoker and chronic-smoker experiment, respectively. After smoking, blood and urine samples were collected for 4 and 20 h after smoking start, respectively. THC blood concentrations reached 2.7 and 4.5 ng/mL in the naive and chronic user, respectively. In both cases, the blood THC concentration is significantly above the Swiss road traffic threshold of 1.5 ng/mL. Consequently, the user was legally unfit to drive directly after smoking. CBD blood concentrations of 45.7 and 82.6 ng/mL were reached for the naive and chronic user, respectively. During the 10-day smoking period, blood and urine samples were regularly collected. No accumulation of any cannabinoid was found in the blood during this time. Urinary 11-nor-9-carboxy-THC concentrations seemed to increase during the 10-day period, which is important in abstinence testing.

1. Introduction

Recently, there has been an increasing interest in cannabinoids for medical applications. Of the many phytocannabinoids, cannabidiol (CBD) has lately been the target of much research as it is non-intoxicating and has potential positive effects on various diseases such as cancer [1,2], epilepsy [3–5] and neurological diseases such as Parkinson's or Alzheimer's disease [6]. CBD has been reported to possess mild sedative effects while reducing negative delta-9-tetrahydrocannabinol (THC) effects such as anxiety [7]. The alteration of the THC-pharmacodynamics has been assigned to negative allosteric modulation of the CB_1-receptor [8]. For a review of potential therapeutic applications of CBD we refer to Pisanti et al. [9]

The consumption, sale and cultivation of cannabis sativa are forbidden or strictly regulated, in most countries [10]. According to the 2017 UN World Drug Report, cannabis is the most consumed illicit drug in the world, with an estimated 183 million users [10]. In the European Union (EU) and Switzerland, the classification of hemp as fiber or drug hemp is linked to its THC content. Swiss law considers hemp or hemp products to be drugs of abuse if the total concentration of tetrahydrocannabinol (THC) and tetrahydrocannabinolic acids (THCA) in the plant material or product is equal or higher than 1% (Appendix A, BetmVV-EDI) [11]. In the EU, the threshold is 0.2% [12].

In Switzerland, the sale of non-medical products containing cannabidiol has seen a massive increase in the past year. Hemp plants containing less than 1% THC and ca. 3–20% CBD are being sold in Switzerland for smoking as a tobacco replacement, as lifestyle products or with no declared use [13]. Although it is legal to produce, sell, possess and to consume these products, the Swiss Federal Office of Public Health strongly recommends that people consuming these products do not participate in traffic [13]. In principle, there is a zero-tolerance policy towards any drug of abuse while driving in Switzerland. In practice, the relevant ordinance of the Swiss Federal Roads Office [14] considers drivers with blood THC concentrations exceeding 1.5 ng/mL as momentarily unfit to drive. To this, a harmonized imprecision of ±30% is applied, giving a cut-off of 2.2 ng/mL. Although 1% THC is a low concentration in plant material, it could be enough to achieve blood concentrations above 2.2 ng/mL. Since the first appearance of CBD-rich products (hereinafter referred to as CBD) on the Swiss market, consumption of such products is often claimed as causative as to the THC concentrations detected in blood.

* Corresponding author.
E-mail address: ulf.meier@bs.ch (U. Meier).

https://doi.org/10.1016/j.forsciint.2018.08.009
0379-0738/

This single case study, conducted as a self-experiment by one of the authors, aimed to investigate whether smoking a CBD joint is enough to achieve THC blood concentrations above the cut-off for driving in Switzerland in both single use (drug-naive) and in simulated chronic use. The results of this study are of particular importance in the interpretation of cases in which the driver claims to have only consumed CBD joints.

2. Materials and methods

2.1. Chemicals and preparation of stock solutions

THC, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC–COOH), 11-hydroxy-delta-9-tetrahydrocannabinol (THC–OH), CBD and cannabinol (CBN) at 1 mg/mL and THC-d3, THC–COOH-d9, THC–OH-d3, CBD-d3 and CBN-d3 at 0.1 mg/mL were purchased from Lipomed AG (Arlesheim, Switzerland). A reference mix containing 0.2 ng/μL CBN/CBD, 0.5 ng/μL THC/THC–OH and 5.0 ng/μL THC–COOH in methanol (MeOH) was prepared. An internal standard mix containing 0.5 ng/μL THC-d3, THC–OH-d3, CBD-d3, CBN-d3 and 5 ng/μL THC–COOH-d9 in MeOH was prepared. Ethyl acetate (EtOAc) in analytical grade was purchased from Roth (Arlesheim, Switzerland). Analytical grade water, MeOH and acetonitrile (ACN) were obtained from Macherey-Nagel AG (Oensingen, Switzerland). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) synthesis grade and β-glucuronidase were purchased from Sigma Aldrich (Buchs, Switzerland). Potassium dihydrogen phosphate in analytical grade, sodium hydroxide pellets and glacial acetic acid (100%) were obtained from Merck (Zug, Switzerland). Certified quality control (QC) blood lyophilisate with a concentration of 2.92 ng/mL THC, 43.1 ng/mL THC–COOH and 2.25 ng/mL THC–OH after reconstitution was obtained from ACQ Science (Rottenburg-Hailfingen, Germany).

2.2. Study design

The study volunteer was a 37 years old female weighing 69 kg (BMI 23.6), non-smoker, who had not consumed any cannabinoid containing products prior to the study.

In the first experiment (hereinafter referred to as naive-smoker experiment), the blood and urine cannabinoid concentrations of the naive volunteer after a single CBD joint were investigated. A CBD joint was smoked over a period of approximately 10 min. The smoke was deeply inhaled and held in the lungs for a few seconds prior to exhaling. Blood samples were taken before smoking, immediately after finishing the joint and then approximately every ten minutes until one hour after the start of smoking. Afterwards, blood samples were collected approximately every 30 min until 4 h after the start of smoking. Blood samples were drawn using a peripheral venous catheter and collected in Vacutainer® (Becton Dickinson AG, Allschwil, Switzerland) tubes with sodium fluoride/potassium oxalate stabilization. Urine samples were collected prior to the start of smoking and from every void for 20 h after smoking. Approximately 30 min after smoke cessation, a DrugWipe 6S (Securetec Detektions-Systeme AG, Neubiberg, Germany) test with oral fluid was done.

The blood and urine cannabinoid concentrations of a modeled chronic user after a CBD joint were investigated. For this, a 10-day smoking period was commenced 35 days after the naive-smoker experiment, at which point the participant was again considered naive. During this period, the participant smoked 2 joints each day, giving a total of 20 joints. Before and after smoking the first daily joint a urine sample was collected. On 5 days during the 10-day smoking-period, blood samples were collected shortly before and approximately 30 min after smoking the second daily joint (approximately 6–8 h after the first joint). When smoking the last joint of the 10-day smoking period the second experiment was conducted (hereinafter referred to as chronic-smoker experiment) and the same sampling scheme as in the naive-smoker experiment was repeated (excluding the DrugWipe test).

All blood and urine samples were stored at 4 °C until analysis. Blood samples were analyzed within one week and urine samples within two weeks after sampling.

2.3. Cannabis material

For the naive-smoker experiment, the CBD cannabis product "Indoor Haze" was purchased from Hempner (Hanfkultur GmbH, Fisibach, Switzerland). For the chronic-smoker experiment, a mixed sample of CBD cannabis, obtained from seizures in local CBD-shops, was used, as "Indoor Haze" was no longer available for purchasing. The mixed sample was finely homogenized before splitting into single portions. The cannabinoid content of the plant material was measured in-house using a validated GC–MS method for cannabis plant material to confirm the declared content and to determine the content of the mixed sample. THC-A and CBD-A were converted directly to THC and CBD in the injector of the GC, giving THC_{total} and CBD_{total}. The declared content of "Indoor Haze" was 23% CBD and 0.8% THC. This was confirmed with measured concentrations of 23.5% CBD_{total} and 0.94% THC_{total}. The cannabinoid content of the mixed sample was measured as 17% CBD_{total} and 0.8% THC_{total}.

Joints contained 500 mg tobacco and 200 mg CBD cannabis, which corresponded to 47 and 34 mg CBD and 1.9 and 1.6 mg THC in the naive-smoker and chronic-smoker experiments, respectively.

2.4. Sample preparation

Blood samples were allowed to reach room temperature and 0.50 (±0.01) g were transferred to a centrifuge tube, diluted with 0.5 mL water and spiked with 6 μL internal standard mix. Protein precipitation was done by addition of 2 mL ACN while vortexing. Samples were centrifuged for 5 min at 3000 rpm. The supernatant was transferred to a headspace vial, further diluted with 3 mL of water and added to the autosampler for the automated sample preparation. Every 15 samples, a QC sample with 0.5 g of reconstituted control blood lypophilisate was prepared. CBN and CBD (different Lot. number than for the calibration) were added to the control blood prior to sample preparation, resulting in a final sample concentration of 0.6 ng/mL for both analytes.

Urine samples were allowed to reach room temperature. A centrifuge tube was filled with 1.00 (±0.01) g of urine and spiked with 6 μL internal standard mix to which 1 mL phosphate buffer (0.1 M, ph 6.8) and 25 μL β-glucuronidase (E. coli, 500 units) were added. The sample was enzymatically hydrolyzed for 16 h at 37 °C. After cooling to room temperature, 2 mL ACN were added while vortexing. After centrifugation, the supernatant was transferred to a headspace vial, diluted with 3 mL water, and placed on the autosampler for the automated sample preparation.

2.5. Automatic online sample preparation

Solid phase extraction (SPE) was performed online on a MultiPurposeSampler 2 (Gerstel GmbH, Mülheim an der Ruhr, Germany) using Chromabond C18 ec polypropylene cartridges (1 mL/100 mg, Macherey-Nagel, Oensingen, Switzerland). The SPE cartridge was conditioned with 2 mL MeOH, followed by 2 mL water, and finally with 1 mL 0.1 M acetic acid. The SPE cartridge was loaded with 5 mL of the pre-treated sample. The cartridge was washed with 1 mL 0.1 M acetic acid, 1 mL ACN:water 2:3 and was dried under nitrogen. The cartridge was eluted with 1.4 mL ACN.

The eluate was evaporated to dryness and 30 μL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide and 20 μL dry EtOAc were added. Sample derivatization was carried out at 90 °C for 17 min.

2.6. GC–MS method

A Trace GC Ultra gas chromatograph was coupled to a TSQ Quantum mass spectrometer (both Thermo Fischer Scientific, Waltham, USA). Separation was achieved with an Optima 5 MS column (30 m, 0.25 mm ID, 0.25 μm film thickness, Macherey-Nagel, Oensingen, Switzerland).

Analysis was done by injecting 2 μL of sample in PTV splitless mode with an injection temperature of 70 °C increased to 250 °C with 14.5 °C/s. The carrier gas (He) flow through the column was 1.5 mL/min. The GC temperature program was as follows: initial temperature 70 °C held for 0.5 min, increased to 200 °C at 80 °C/min, increased to 300 °C at 10 °C/min and held for 3 min for a total run time of 15 min. The transfer line temperature was set to 250 °C. The selected reaction monitoring (SRM) conditions and retention times are shown in Table 1. The filament emission current was set to 50 μA with a source temperature of 250 °C. Data acquisition, instrument control and data evaluation were performed with Thermo Xcalibur (version 2.1.0.1140, Thermo Fischer Scientific, Waltham, USA).

2.7. Urine immunoassay testing

All urine samples were tested with the Indiko™ Plus immunoassay (Cannabinoids Multi Level THC CEDIA test kit, Thermo Fischer Scientific) using a cut-off of 50 ng/mL for THC–COOH prior to GC–MS/MS analysis.

2.8. Urine creatinine normalization

To better compare the urine samples, the analyte concentrations were normalized to the creatinine content of the urine. Creatinine normalization was done as described by Cone et al. [15]:

$$Conc._{Norm} = \frac{Conc._{Sample}}{CR_{Sample}} * CR_{Ref} \quad (1)$$

where CR_{Ref} is a reference concentration chosen as 100 mg/dL, which is close to the mean of all samples of the volunteer of 92 mg/dL, $Conc._{Sample}$ is the analyte concentration [ng/mL] in the sample, CR_{Sample} is the creatinine concentration [mg/dL] in the sample and $Conc._{Norm}$ is the creatinine normalized concentration [ng/mL] of the sample.

3. Results and discussion

3.1. Validation parameters

The method was validated according to the guidelines of the Swiss Society of Legal Medicine and has proven its suitability for forensic–toxicological routine work by successful application in proficiency tests. The validation parameters can be found in Table 2.

3.2. Cannabis rapid screening test

The DrugWipe saliva test yielded a negative result. According to the manufacturer, the DrugWipe 6S test has a detection limit of 5 ng/mL for THC and no cross reactivity with CBD.

3.3. Blood cannabinoid concentrations

The cannabinoid blood concentration profiles of the naive-smoker and chronic-smoker experiments are shown in Fig. 1. The numerical results can be found in the Supplementary material. All time points are given relative to smoking start (t = 0 min).

3.3.1. Naive-smoker experiment

No cannabinoids were detected in the blood sample taken prior to smoking in the naive-smoker experiment. After smoking, a maximum CBD concentration of 45.7 ng/mL was found in the first blood sample at ca. 10 min. The concentrations remained above the LOQ during the entire collection time with a concentration of 0.9 ng/mL at 4.5 h. The highest THC concentration, 2.7 ng/mL, was also found in the first blood sample after smoking. The concentration quickly declined, dropping below the decision cut-off (2.2 ng/mL) in the second blood sample taken at ca. 20 min and below the LOD (0.2 ng/mL) in the fourth sample taken at ca. 40 min. No THC–OH (LOD = 0.2 ng/mL) or THC–COOH (LOD = 2.0 ng/mL) was detected in any sample. CBN was detected in the first blood sample after smoking at a concentration below the LOQ (0.3 ng/mL)

3.3.2. 10-day smoking period

On 5 days of the 10-day smoking period leading up to the chronic-smoker experiment blood samples were collected before and after smoking the second daily joint. The results of these samples are shown in Table 3. Starting from the second day of the 10-day period some CBD could be found in every blood sample taken before smoking with concentrations in the range of ca. 0.4–

Table 1
SRM conditions and analyte retention times.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Collision energy (eV)
THC	371.2	289.3	9.0	20
	386.3	371.4		13
THC–OH	371.3	289.2	10.9	15
THC–COOH	371.3	289.2	11.9	15
	488.3	398.3		15
CBN	367.4	295.2	9.7	30
	367.4	310.2		20
CBD	390.2	231.05	8.1	20
	390.2	300.8		10
THC-d3	374.3	292.2	9.0	20
	389.3	374.4		13
THC–OH-d3	374.3	292.3	10.9	15
THC–COOH-d9	380.3	292.2	11.8	15
	497.3	380.4		15
CBN-d3	370.5	295.3	9.7	30
	370.5	310.3		25
CBD-d3	393.07	304.53	8.1	10

Table 2
Validation parameters of the cannabinoids. The method was validated for blood. Selectivity and linearity were verified for urine. Application of the method in routine use has shown that validation parameters obtained for blood are transferrable to urine analysis. Signal to noise ratios (S/N) were used to determine LOD (S/N ≥ 3) and LOQ (S/N ≥ 10) based on of the least abundant transition in fortified blank serum samples with the additional acceptance criteria that the calculated concentration must be within ±20% of the nominal value and imprecision lower than ±20%.The expanded measurement uncertainty was estimated using 50 blood control samples over a one year period.

Analyte	Linearity (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Extraction efficiency (%)	Accuracy bias (%)	Expanded measurement uncertainty (%) (2 × CV%)
THC	0.5–20	0.2	0.3	113 ± 5	2.7	18
THC–OH	0.5–20	0.2	0.3	95 ± 2	−1.3	20
THC–COOH	5–200	2.0	3.0	105 ± 7	−10.3	23
CBN	0.5–50	0.2	0.3	90 ± 10	−10.3	41
CBD	0.5–100	0.2	0.3	88 ± 13	−6.7	38

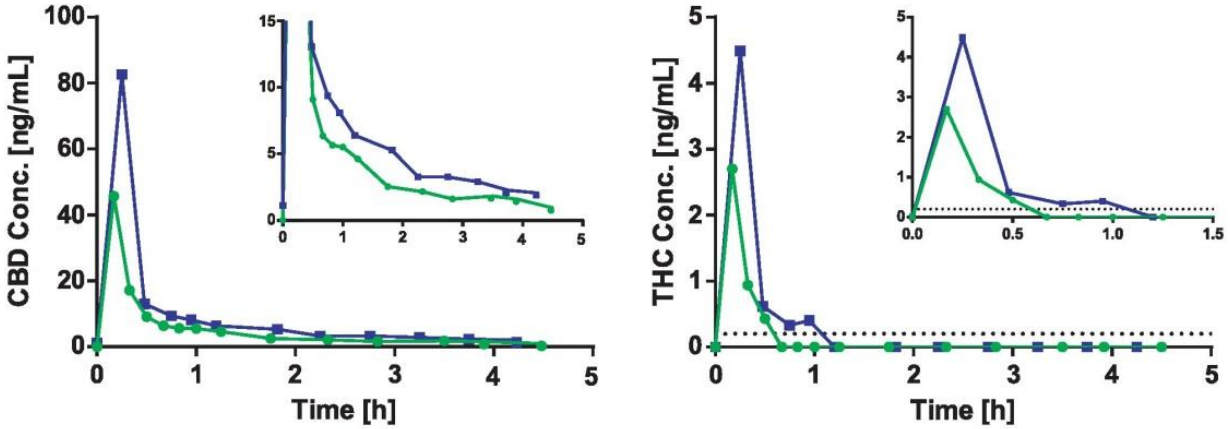


Fig. 1. CBD (left) and THC (right) blood concentrations obtained for the naive-smoker (circles) and chronic-smoker (squares) experiments. The dotted line shows the limit of detection (0.2 ng/mL). The smaller panels within the graphs show enlarged areas of interest.

Table 3
CBD and THC concentrations in the blood samples collected before and after smoking the second daily joint during the 10-day smoking period.

Day	Time between smoking start and sampling (min)	CBD before/after smoking (ng/mL)	THC before/after smoking (ng/mL)
2	20	1.3/13.7	<LOD/0.7
3	25	1.2/15.0	<LOD/0.6
4	30	0.6/11.5	<LOD/0.7
8	30	ca. 0.4/11.7	<LOD/0.5
9	30	0.5/10.5	<LOD/<LOD
Mean	27	0.8/12.5	–/0.5[a]
StD Dev	–	0.4/1.8	–/0.3[a]

[a] Mean and StD Dev calculated with <LOD = 0 ng/mL.

1.3 (mean 0.8 ± 0.4) ng/mL but no trend in the concentration was observed. THC could not be detected in any sample taken directly before smoking. THC was detectable in the samples taken at 20–30 min after smoking in 4 of 5 samples with concentrations between 0.5 and 0.7 ng/mL. The obtained CBD concentrations after smoking were between 10.5 and 15 (mean 12.5 ± 1.8) ng/mL. THC–COOH,THC–OH, and CBN were not detectable in any blood sample collected before or after smoking.

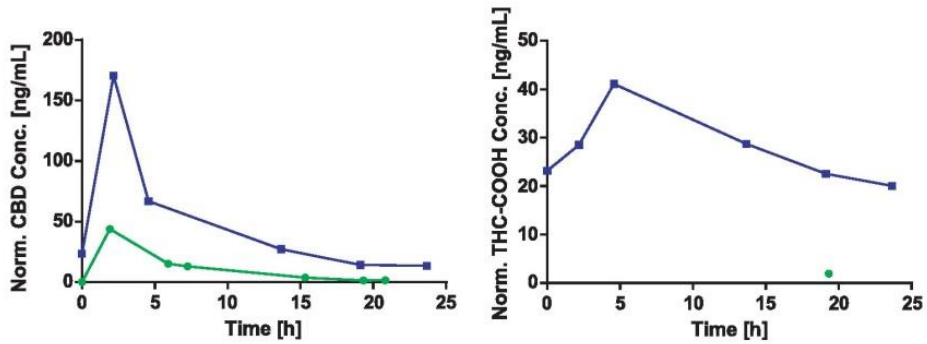


Fig. 2. Creatinine normalized CBD (left) and THC–COOH (right) urine concentrations obtained for the naive-smoker (circles) and chronic-smoker (squares) experiments.

3.3.3. Chronic-smoker experiment

The CBD concentration in the blood sample taken prior to smoking the last joint of the 10-day smoking period and thereby commencing the chronic-smoker experiment was 1.1 ng/mL. No other cannabinoid was detected in this blood sample. After smoking, a maximum CBD concentration of 82.6 ng/mL was found in the first blood sample withdrawn at ca. 15 min. CBD concentration remained above the LOQ for the entire collection period of 4.25 h with a final concentration of 2.1 ng/mL. The highest THC concentration of 4.5 ng/mL was also obtained in the first blood sample after smoking. THC concentrations dropped below the decision cut-off in the second blood sample at ca. 30 min and below the LOD in the fifth sample at ca. 70 min. A THC–OH concentration of ca. 0.3 ng/mL was found in first blood sample. THC–OH concentration in the second sample at ca. 30 min was still above the LOD. No THC–OH could be detected in the following samples. CBN was only detected in the first blood sample after smoking with a concentration of 0.8 ng/mL. THC–COOH could not be detected in any sample.

3.4. Urine cannabinoid concentrations

Urine results of the naive-smoker and chronic-smoker experiments are shown in Fig. 2. All urine concentrations are given as creatinine normalized concentrations. All time points are given relative to smoking start (t = 0 min).

3.4.1. Naive-smoker experiment

For the naive-smoker experiment, no cannabinoids were detected in the urine before smoking. A maximum CBD concentration of 44.0 ng/mL was observed in the first urine at 1.9 h. In each subsequent urine the concentrations continuously decreased. A CBD concentration of 1.7 ng/mL was found in the last urine sample given at 20.8 h. THC–COOH was only detectable in one urine sample, 19.3 h after smoking, at a concentration of 1.9 ng/mL (non-normalized ca. 3.6 ng/mL). This sample showed a much higher (factor three) creatinine concentration than any other urine sample, suggesting concentrated urine. This is likely the reason THC–COOH could be detected in only this sample. No THC was found in any urine sample.

3.4.2. 10-day smoking period

The results of the urine samples taken before and after smoking the first joint each day during the 10-day smoking period are shown in Fig. 3. CBD could be detected in every sample with a maximum concentration of 219 ng/mL. A maximum THC–COOH concentration of 42.5 ng/mL was found on the eighth day. The samples marked with a large circle in Fig. 3 gave positive results with the immunoassay test.

3.4.3. Chronic-smoker experiment

The urine sample taken prior to smoking the last joint of the 10-day period and thereby commencing the chronic-smoker experiment showed CBD and THC–COOH concentrations of 23.4–23.2 ng/mL, respectively. After smoking, a maximum CBD concentration of 171 ng/mL was found at 2.2 h. The highest THC–COOH concentration was found at 4.6 h with 41.1 ng/mL. No THC was found in any sample.

4. Discussion

The concentration profiles of THC and CBD were similar to each other as well as being similar in both the naive-smoker and chronic-smoker experiments. Both CBD and THC showed a sharp increase in concentration immediately after smoking, followed by a rapid decrease from distribution of the cannabinoids into the tissues, and finally a very slow elimination from blood. The observed biphasic pharmacokinetics of CBD and THC are in line with the profiles described in the literature [16,17].

In Switzerland, the limit according to the relevant ordinance [14] for THC in whole blood is 1.5 ng/mL for driving. At blood levels $\geq$1.5 ng/mL the driver is defined as momentarily unfit for driving, regardless of whether or not the accused presents typical cannabis-related symptoms or shows inappropriate driving maneuvers. Considering the harmonized imprecision used in Switzerland of 30% results in a decision threshold value of 2.2 ng/mL. The THC blood concentrations of the participant rose above this limit in both experiments, after which the concentrations dropped below 2.2 ng/mL at around 30 min after smoking start. Thus, individuals smoking CBD cigarettes or joints directly prior to or during driving could be unfit for driving according to the Swiss Road Traffic Act and risk losing their driver's license if they are subjected to a control by the police. Assuming similar concentration-time profiles as seen in this study, only blood samples collected shortly after CBD consumption could result in THC blood levels above the legal limit. Usually, there is some time between being stopped by the police and the collection of blood specimens. Therefore, the risk of losing the driver's license due to smoking a CBD joint is relatively low. However, as blood levels can exceed the limit for THC, we fully support the recommendation by the Swiss Federal Office of Public Health to not smoke CBD joints prior to driving.

THC did not show any accumulation during the 10-day smoking period. Smoking experiments comparing cannabinoid concentrations in whole blood of occasional and regular cannabis smokers showed significant THC accumulation in regular cannabis users [18]. However, it seems that the consumed amount of THC in the present study was too low to give detectable accumulation of THC. If more or stronger joints were smoked per day or if smoking would be continued for a longer time period, it cannot be ruled out that some

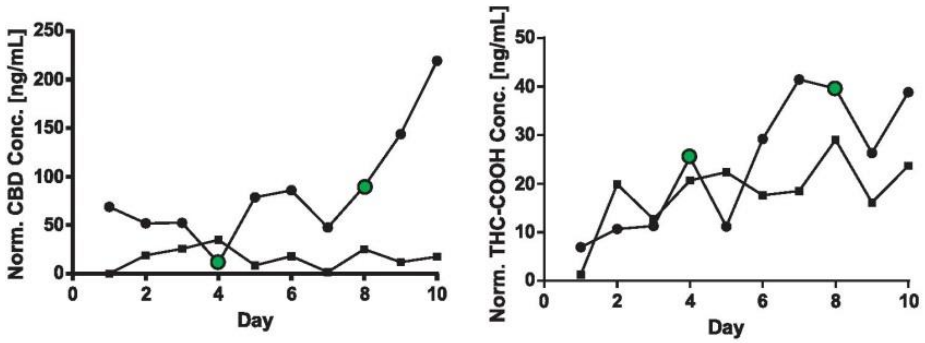


Fig. 3. Creatinine normalized CBD (left) and THC–COOH (right) urine concentrations obtained in the urine samples taken before (squares) and after (circles) smoking the first joint of each day during the 10-day smoking period. The large circles show samples which tested positive using the immunological test with a THC–COOH cut-off of 50 ng/mL.

accumulation might occur. Low levels of CBD were found in blood samples taken prior to smoking the second daily joint during the 10-day smoking period. However, there was no continuous CBD concentration increase during this period, although such a trend might be obscured by the variation in sampling times after smoking.

As is visible in Fig. 3, there seems to be a trend of increasing urinary THC–COOH concentrations during the 10-day smoking period. This could be relevant for cannabinoid testing in urine e.g. in cannabis abstinence testing. Urine cannabinoid concentrations are difficult to interpret and a conclusive differentiation between CBD- or THC-rich cannabis consumption might not always be possible.

The mayor limitation of this study is that it was only conducted with a single person. Therefore, the presented results should be carefully interpreted as inter-individual differences can be expected. Studies with more participants, over a longer time period and with a more frequent sampling scheme are needed to give more concrete recommendations for driving fitness and for providing a basis to differentiate between licit and illicit cannabis consumption. Additionally, sampling frequency during the adsorption phase was most likely too low to capture the maximal blood concentration. Especially in the minutes directly after smoking start, more frequent sampling would have been needed to describe the initial phase sufficiently.

5. Conclusion

The presented case study shows that immediately following smoking of a CBD rich joint (THC below 1%), THC concentrations above the legal limit for driving in Switzerland can be reached. Therefore, CBD smokers should refrain from driving for some hours after smoking. No accumulation of THC was seen when smoking 2 joints per day over a 10-day period.

6. CRediT authorship contribution statement

Ulf Meier: Conceptualization, Investigation, Writing – Original Draft, Visualization. **Franz Dussy:** Conceptualization, Investigation, Writing – Review & Editing. **Eva Scheurer:** Writing – Review & Editing. **Katja Mercer-Chalmers-Bender:** Writing – Review & Editing. **Sarah Hangartner:** Validation, Conceptualization, Methodology, Investigation, Writing – Review & Editing, Project Administration.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

We would like to sincerely thank Elke Dussy, Anne-Catherine Kessler, Andrea Oswald, Nathalie Schwab, and Christine Schaffer for their work in taking the blood samples. We would also like to thank the two lab technicians Theresa Otz-Rüttimann and Cornelia Hamberg for their diligent work doing the sample preparations of the blood and urine samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2018.08.009.

References

[1] R. Ramer, S. Fischer, M. Haustein, K. Manda, B. Hinz, Cannabinoids inhibit angiogenic capacities of endothelial cells via release of tissue inhibitor of matrix metalloproteinases-1 from lung cancer cells, Biochem. Pharmacol. 91 (2) (2014) 202–216.
[2] A. Ligresti, A.S. Moriello, K. Starowicz, et al., Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma, J. Pharmacol. Exp. Ther. 318 (3) (2006) 1375–1387.
[3] O. Devinsky, J.H. Cross, L. Laux, et al., Trial of cannabidiol for drug-resistant seizures in the dravet syndrome, N. Engl. J. Med. 376 (21) (2017) 2011–2020.
[4] O. Devinsky, E. Marsh, D. Friedman, et al., Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial, Lancet Neurol. 15 (3) (2016) 270–278.
[5] E.C. Rosenberg, R.W. Tsien, B.J. Whalley, O. Devinsky, Cannabinoids and epilepsy, Neurotherapeutics 12 (4) (2015) 747–768.
[6] T. Iuvone, G. Esposito, D. De Filippis, C. Scuderi, L. Steardo, Cannabidiol: a promising drug for neurodegenerative disorders? CNS Neurosci. Ther. 15 (1) (2009) 65–75.
[7] A.W. Zuardi, I. Shirakawa, E. Finkelfarb, I.G. Karniol, Action of cannabidiol on the anxiety and other effects produced by delta 9-THC in normal subjects, Psychopharmacology (Berl) 76 (3) (1982) 245–250.
[8] R.B. Laprairie, A.M. Bagher, M.E. Kelly, E.M. Denovan-Wright, Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor, Br. J. Pharmacol. 172 (20) (2015) 4790–4805.
[9] S. Pisanti, A.M. Malfitano, E. Ciaglia, et al., Cannabidiol: state of the art and new challenges for therapeutic applications, Pharmacol. Ther. 175 (2017) 133–150.
[10] UNODC, World Drug Report 2017, United Nations Publication, 2017.
11 Eidgenössisches Departement des Innern. Betäubungsmittelverzeichnisverordnung. Admin.ch. https://www.admin.ch/opc/de/classified-compilation/20101220/index.html. Published May 2011. (Accessed 22 March 2018).
[12] EMCDDA, Cannabis Legislation in Europe: An Overview, Publications Office of the European Union, Luxembourg, 2017.
[13] BAG, BLV, BLW, Produkte Mit Cannabidiol (cbd) — Überblick Und Vollzugshilfe, Schweizerische Eidgenossenschaft, 2017.
[14] Der Eidgenössische Bundesrat. Verordnung über die Betäubungsmittelkontrolle. Admin.ch. https://www.admin.ch/opc/de/classified-compilation/20101221/index.html. Published Mai 2011. (Accessed 22 March 2018).
[15] E.J. Cone, Y.H. Caplan, F. Moser, T. Robert, M.K. Shelby, D.L. Black, Normalization of urinary drug concentrations with specific gravity and creatinine, J. Anal. Toxicol. 33 (1) (2009) 1–7.
[16] M.A. Huestis, A.H. Sampson, B.J. Holicky, J.E. Henningfield, E.J. Cone, Characterization of the absorption phase of marijuana smoking, Clin. Pharmacol. Ther. 52 (1) (1992) 31–41.
[17] A. Ohlsson, J.E. Lindgren, S. Andersson, S. Agurell, H. Gillespie, L.E. Hollister, Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration, Biomed. Environ. Mass Spectrom. 13 (2) (1986) 77–83.
[18] M. Fabritius, H. Chtioui, G. Battistella, et al., Comparison of cannabinoid concentrations in oral fluid and whole blood between occasional and regular cannabis smokers prior to and after smoking a cannabis joint, Anal. Bioanal Chem. 405 (30) (2013) 9791–9803.

Table S.I. 1 Blood cannabinoid concentrations measured during the naive-smoker experiment. All time points are given relative to smoking start (t=0).

Sample	Time [h]	CBD [ng/mL]	THC [ng/mL]	CBN [ng/mL]	THC-OH [ng/mL]	THC-COOH [ng/mL]
0	0.00	<LOD	<LOD	<LOD	<LOD	<LOD
1	0.17	45.7	2.7	<LOQ	<LOD	<LOD
2	0.33	17.2	0.9	<LOD	<LOD	<LOD
3	0.50	9.1	ca. 0.4	<LOD	<LOD	<LOD
4	0.67	6.3	<LOD	<LOD	<LOD	<LOD
5	0.83	5.6	<LOD	<LOD	<LOD	<LOD
6	1.00	5.5	<LOD	<LOD	<LOD	<LOD
7	1.25	4.6	<LOD	<LOD	<LOD	<LOD
8	1.75	2.5	<LOD	<LOD	<LOD	<LOD
9	2.33	2.2	<LOD	<LOD	<LOD	<LOD
10	2.83	1.6	<LOD	<LOD	<LOD	<LOD
11	3.50	1.8	<LOD	<LOD	<LOD	<LOD
12	3.92	1.6	<LOD	<LOD	<LOD	<LOD
13	4.50	0.9	<LOD	<LOD	<LOD	<LOD

Table S.I. 2 Blood cannabinoid concentrations measured for the chronic-smoker experiment. All time points are given relative to smoking start (t=0).

Sample	Time [h]	CBD [ng/mL]	THC [ng/mL]	CBN [ng/mL]	THC-OH [ng/mL]	THC-COOH [ng/mL]
0	0.00	1.1	<LOD	<LOD	<LOD	<LOD
1	0.25	82.6	4.5	0.8	ca. 0.3	<LOD
2	0.48	13.0	0.6	<LOD	<LOQ	<LOD
3	0.75	9.4	ca. 0.3	<LOD	<LOD	<LOD
4	0.95	8.1	ca. 0.4	<LOD	<LOD	<LOD
5	1.20	6.4	<LOD	<LOD	<LOD	<LOD
6	1.82	5.3	<LOD	<LOD	<LOD	<LOD
7	2.25	3.3	<LOD	<LOD	<LOD	<LOD
8	2.75	3.3	<LOD	<LOD	<LOD	<LOD
9	3.25	2.9	<LOD	<LOD	<LOD	<LOD
10	3.75	2.3	<LOD	<LOD	<LOD	<LOD
11	4.25	2.1	<LOD	<LOD	<LOD	<LOD

4. Discussion, Conclusion, and Outlook

4.1. The Path of the Project

Errors during sample collection are hard to deal with. Much effort is spent on developing and validating analytical methods and quantifying accuracy, precision, extraction yields, matrix effects, etc. The process of collecting the sample is often neglected and the impact thereof underestimated. If a sample is collected that doesn't represent the target of the investigation, the measurement will not yield correct results. This project is aimed at investigating the crucial step of sample collection during hair analysis, and whether a lock of head hair from any given location is representative for the entirety of the scalp hair.

The overarching path of this project can be described as follows. Project 1 was an exploratory study to investigate whether sampling location dependent concentration differences and distribution patterns exist and whether the subject was worth a more thorough investigation. As the project yielded interesting results, the second study was designed to expand on the first study by including many test subjects and expanding the number of investigated substances to include DoA and BZD-Z and to increase the EtG sample size. As it was suspected that such a study would involve many samples, a combined sample preparation method for EtG, DoA, and BZD-Z was developed. With the combined sample preparation method in hand, the study was commenced, and 13 participants were recruited. The results of Project 3 supported that sweating rates could be an important factor influencing the distribution patterns. Therefore, the plausibility of differences in sweat rates being the main factor determining the distribution patterns was investigated by taking a closer look at the incorporation of substances into hair from sweat. As one study participant reported consuming Kratom, an analysis method for the main psychoactive alkaloids mitragynine and 7-Hy-Mitra was developed and the distribution patterns were investigated.

The project investigating the cannabinoid concentrations in blood and urine after smoking CBD joints had nothing to do with the subject of the thesis. Instead, it was done as there was an urgent need for information concerning if smoking CBD hemp could cause blood concentrations to reach the Swiss legal cut-off value of THC for driving.

In the following chapters, the results of the individual projects are summarized and generally discussed, before discussing their relevance in routine forensic application, recommendations for hair sampling, and limitations of the studies.

4.2. Summarized Results

In the following, the results of the individual projects are very briefly summarized. For a more extensive presentation of the results including tables, graphs and figures see the corresponding sections.

The study participant of project 1 showed sampling location dependent EtG and caffeine concentrations in his scalp hair. Caffeine concentrations ranged from 1.1 ng/mg to 12.0 ng/mg, meaning a factor of 10.6 difference between the lowest and highest concentration. EtG concentrations ranged from 6.8 pg/mg to 20.2 ng/mg, resulting in a factor of 3.0. The caffeine distribution pattern showed higher concentrations towards the edges of the haircut (especially on the forehead) and lowest concentration in the vertex region. EtG showed lower concentrations at the back of the neck, but otherwise no clear pattern was identified.

In project 2, a method for the combined sample preparation of hair samples for subsequent analysis for EtG and DoA with LC-MS was successfully developed and fully validated. The method facilitated the measurement of the large number of samples of project 3.

Project 3 was the main work of this thesis and yielded by far the most extensive results. Thirteen alcohol and/or cocaine consuming participants were recruited for the study. Adding the results of the first project yielded a sample size of 10 for EtG and 12 for cocaine. Most of the participants consumed many substances and distribution patterns of 29 substances were obtained. The factors between minimum and maximum cocaine concentrations of the individual participants ranged from 2.8 up to 105 with a mean factor of 17.6. Large concentration differences were not unique to cocaine, and factors above 10 could often be observed for many of the investigated substances. The distribution patterns of cocaine, most other DoA, and some BZD-Z were similar to the pattern observed for caffeine in project 1, with higher concentrations on the periphery of the scalp. This distribution shape was found for all participants (except for one) and was very similar across participants, although the extent of the concentration differences was very substance and participant dependent. EtG also showed relevant differences across the scalp, with factors between minimum and maximum concentrations of the individual study participants ranging from 2.5 to 7.1, with a mean factor of 4.4. The distribution of EtG was often reverse to that of cocaine, with higher concentrations in the vertex region. The lowest EtG concentrations were usually found at the back of the neck. To describe the distribution pattern, each substance was compared with cocaine using Spearman correlations to determine how “cocaine-like” the distributions were. Most DoA and BZD-Z showed distribution patterns very similar to the distribution shown by cocaine. The perfusion measurements yielded no clear patterns, except for lower perfusion values towards the back of the neck. Correlating the perfusion values with the EtG and cocaine concentrations yielded statistically significant correlations in half of the cases. In cases with significant correlations, the correlation coefficient was positive for EtG and negative for cocaine, signifying that higher perfusion rates are correlated to higher EtG and lower cocaine concentrations. The correlation was not very strong however, so the perfusion is likely not a main contributor to the distribution patterns. Sweat rate measurements yielded a recurring pattern of higher sweat rates at the forehead, and generally higher on the periphery of the scalp, decreasing towards the vertex. While a direct correlation of sweating results with the concentrations was not possible, the pattern of sweating was visually similar to the distribution patterns observed for the DoA/BZD-Z. Consequently, differences in sweat rates could be a main contributor to the concentration distribution patterns and most DoA and

BZD-Z could be incorporated into the hair from sweat, while EtG is more likely washed out of the hair by the sweat.

No definite results can be presented yet for the incorporation of substances into hair from spiked artificial sweat solutions. The preliminary results show that all substances can enter the hair from spiked sweat solutions. The concentrations found in or on the hair are substance dependent. EtG showed the lowest concentrations after soaking of all tested substances. Of the DoA, benzoylecgonine showed the lowest and methadone the highest concentrations.

A method for the measurement of mitragynine and 7-Hy-Mitra in hair was successfully developed and validated according to the guidelines of the GTFCh. Mitragynine concentrations in the hair of the self-reported consumer were between 1.1 ng/mg and 2.2 ng/mg (mean 1.5 ng/mg). 7-Hy-Mitra was not detected in any sample. No clear distribution pattern was observed for mitragynine concentrations. The measurement of 300 hair samples from our routine analysis population did not reveal a single positive result. This shows that the prevalence of Kratom consumption is likely low in our population of mostly people in drivers-license regranting procedures.

Project CBD demonstrated that THC blood concentrations above the legal threshold for driving in Switzerland could be reached shortly after smoking a joint with CBD rich cannabis. Maximum THC concentrations of 2.7 ng/mg and 4.5 ng/mg were reached after the single and chronic smoking experiments, respectively. CBD concentrations reached 45.7 ng/ml and 82.6 ng/ml after the single and chronic smoking experiments, respectively. No accumulation of THC in blood or urine could be detected during the 10-day smoking period. A slight accumulation of THC-COOH in urine is possible.

4.3. General Discussion

Incorporation via the bloodstream, incorporation via sweat or sebum, and incorporation from external sources are considered the three main incorporation pathways of substances into the hair (see section 1.5). It is likely that incorporation of a substance into the hair happens via a combination of these pathways. Concentration differences across the scalp show that the amount being incorporated through one or more of these pathways is scalp-location dependent. As the extent of the concentration differences is substance and participant dependent, it is likely that the portion that each pathway contributes to the concentration in the hair is also substance and participant dependent. As the distribution of the sweat rates is similar to the concentration distribution pattern for the “cocaine-like” substances, it is likely that the sweat rates govern the shape of the distribution. The perfusion rates were lower on the neck but otherwise no pattern was observed across participants. Therefore, differences in the perfusion rates across the head likely do not cause the observed concentration distribution patterns. The following can be hypothesized:
The incorporation through blood is largely uniform across the head while the incorporation via sweat is dependent on local sweat rates and is responsible for concentration differences across the scalp. The portion of the concentration in the hair that each pathway is responsible

for is substance dependent, head location dependent, and influenced by interindividual factors.
If sweat rate differences are responsible for the concentration differences across the head, substances must be able to transitions from the blood into the sweat and then from the sweat into the hair. As the extent of the differences across the head is substance dependent, substances likely differ in their propensity for one or both transitions. Cocaine-like substances that show larger concentration differences across the head would be incorporated into the hair to a larger portion through the sweat. These transition steps should be investigated thoroughly in future studies.
Fairly small changes in the chemical structure can strongly impact the distribution as can be seen from the extent of concentration differences and how cocaine-like morphine and codeine are compared to 6-monoactelymorphin and acetylcodeine. The acetylated substances show more cocaine-like and more extreme concentration differences than their non-acetylated counterparts. Therefore, lipophilicity might play an important role for incorporation via sweat.
For some substances, the extent of differences is highly participant dependent, which could be caused by e.g. differences in sweat pH, hair structure, co-consumption of substances, differences in metabolism, or amount of consumed substance.
The soaking experiments that are currently underway are meant to test the ability of the substances to enter the hair from the sweat. The preliminary results do not contradict this hypothesis. However, the observed concentration differences after 10 soaks do not align with the differences in the extent of concentration distribution across the scalp. Of the DoA, methadone showed some of the smallest differences across the scalp but in the soaking pre-experiments showed the highest hair concentrations while benzoylecgonine generally showed large differences across the scalp but the lowest incorporation of the DoA in the soaking experiment. Therefore, the transition from blood to sweat is likely the dominating factor determining the distribution patterns. A more thorough discussion can be held when definite results from the soaking experiments are available.
Unfortunately, there is little to no literature available on sweat concentrations in relationship to blood concentrations for the investigated substances. The propensity of substances to enter the sweat from the blood should be investigated in future studies and be compared with the extent of the concentration differences across the scalp of the different substances.

4.4. Relevance of the Results for Routine Forensic Applications

The large concentration differences described herein are highly significant for routine applications for many reasons and should be considered during the sampling protocols of laboratories doing routine work. Cut-off values are often applied in forensic hair analysis, especially for abstinence testing for e.g. driving-license regranting. It is the nature of cut-off values that the decisions are binary; either the value is above (positive) or below (negative) a given cut-off. When the concentration of a substance in a hair lock is close to the cut-off, measurement error can cause repeated measurements of that lock to sometimes yield positive and sometimes negative results. While cut-off values are very practical and should allow a fair and reproducible judgement, this is an intrinsic problem of cut-off values. A serious problem arises, if a factor with a large influence on the results, such as sampling location of hair on the

head, is not considered or controlled. In this case, sampling from a different location can cause the result of the test subject to be interpreted completely differently. This is illustrated well by regarding study participant 10 of project 3. This participant showed concentrations of EtG ranging from 6.2 pg/mg up to 30.4 pg/mg. If applying the SoHT recommended cut-offs for EtG of 7 pg/mg (5 pg/mg according to the new SoHT consensus of 06.08.2019[106]) for abstinence and 30 pg/mg for chronic excessive consumption, this participant would be classified as in accordance with a self-declared abstinence (if applying the former cut-off), a social drinker, or a chronic excessive consumer, depending on from where the hair lock was taken. For seven of the ten study participants, EtG concentrations fell into more than one category. A similar thing occurs when applying the SoHT recommended cocaine cut-off value of 500 pg/mg to study participant 7. This participant showed cocaine concentrations ranging from 312 pg/mg to 4773 pg/mg, so would also be judged differently depending on sampling location. As previously stated, measurement errors can cause some values to be below or above a given cut-off when the concentration is close to the cut-off. This is different however, in that 312 pg/mg is not particularly close to the 500 pg/mg cut-off and 4773 pg/mg is 9-fold above the cut-off. These cases clearly illustrate that if hair sampling location is not controlled, the interpretation of results when applying cut-offs is sampling site dependent. Even in cases where cut-offs are not applied, differences across the scalp can lead to different interpretations of the consumption behavior, in the sense that even though it is not possible to calculate the consumed dose of a substance from hair concentrations, many laboratories do classify hair concentrations roughly as e.g. low, mid, or high compared to the concentrations otherwise measured at that laboratory.

In forensics, the ability to confirm results is very important. Usually, at least two locks of hair are sampled; one is measured, and another is kept in reserve for confirming the result in case e.g. the test subject does not accept the result. If these locks of hair are taken from different head areas, the second measurement will not necessarily confirm the first measurement. This is an uncomfortable situation for the forensic toxicologist as they do not know the cause of the discrepancy. It could be a bad calibration, a sample mix-up, or many other things. Even if the toxicologist is aware that the hair locks were taken from different places, large concentration differences could be a hard sell if the results need to be presented to judge or jury in court.

External contamination of hair is a large concern in hair analysis as it can lead to false positive results. A strategy to counter this is to rely on metabolites of a substance and to use metabolite-parent substance ratios to classify a result as external contamination or uptake of a substance. This ratio classification is usually done using a method dependent cut-off. The results of project 3 show that due to the different extents of concentration differences of e.g. cocaine, benzoylecgonine, and norcocaine, the metabolite-parent ratios are also sampling site dependent. The same considerations concerning cut-offs also apply here, and the classification as external contamination or consumption can in some cases be sampling location dependent.

In studies looking at dose-concentration relationships in hair, the linearity is often poor. This is likely due to the many factors affecting the incorporation of substances into the hair, as described in the introduction. If the sampling location was not strictly controlled during these studies, sampling location can be another factor affecting the results. Therefore, if efforts are to be made to ever establish dose-concentration relationships, the sampling location should be considered and controlled.

The results of the soaking experiments of project 4 will be relevant for routine forensic hair interpretation. From the preliminary results it seems that all tested substances enter the hair and can be incorporated strongly enough to not be removed by the applied standard washing procedure. This is problematic for interpretation of results as it opens up the possibility of transfer of substances of a consumer to a non-consumer via sweat, including drug metabolites.

The combined sample preparation method that was developed to deal with the large number of samples of project 3 has proven valuable in routine work. In cases with little available hair, using the combined method, an analysis for EtG and DoA and BZD-Z is possible. Our lab is relatively small, and we do not have many samples for which multiple analyte groups are requested (in 2018: Total ca. 860, EtG ca. 470, DoA + BZD-Z ca. 390, EtG + DoA or BZD 140). Therefore, the combined method is not regularly used. For larger labs with more samples for which a broad range of analytes is requested, this method could greatly reduce sample preparation time and reduce the amount of required hair.

No method for measuring mitragynine and 7-Hy-Mitra in hair had previously been published. Kratom falls under varying legal classifications around the world and the legality of Kratom has seen a lot of change in the last few years. Especially in the USA, Kratom consumption seems to have gained popularity. Therefore, the study on mitragynine distributions in hair of a regular consumer could be useful for toxicologists if the question of detecting Kratom consumption in hair ever arises.

With the legal availability of CBD rich cannabis with THC concentrations below 1% in Switzerland, the question of whether the consumption of CBD cannabis could cause THC levels to rise above the cut-off for driving became urgent. The one-person experiment showed that for a short time after consuming a CBD joint, concentrations above this level could be found in blood. After about 30 minutes, concentrations of THC were again below the cut-off. This is a one-person experiment and more thorough studies are needed to investigate typical concentrations levels of cannabinoids after consuming CBD material. The effects that CBD or the combination of CBD and THC might have on driving capacity need to be investigated. This study can serve as a starting point for planning these studies. In fact, a larger study has recently been commenced at our institute to investigate the effects of CBD on driving capacity.

4.5. Recommendations for Hair Sampling

We recommend following the recommendations of the SoHT and the EWDTS to sample hair from the vertex posterior and to sample adjacent hair locks. Following the current recommendation has three main benefits:
Firstly, the vertex posterior is the established sampling region. Most research on hair analysis has been done in this region, meaning it is the best described area, and the cut-off values were established in parts based on results obtained from this region. The metabolite-parent ratios were also studied in hair from this region. Considering the massive concentration differences described in this study, the cut-offs recommended by e.g. the SoHT should, depending on the

question to be answered, not be applied to hair taken from other regions. At the very least, it should be documented from where the hair was sampled.
Secondly, the data suggest that the concentrations in the vertex area are more homogenous than elsewhere. The concentration gradients are steeper towards the periphery of the scalp. This homogeneity has the large benefit that hair locks taken directly adjacent to each other will have a higher likelihood of containing comparable concentrations. This is very beneficial when results need to be confirmed.
Thirdly, for the analysis of EtG, the vertex is suitable as the concentrations are higher in this area. EtG is a low concentration marker,[107-109] and measuring low EtG concentrations can be analytically demanding. Sampling from another region might necessitate lowering the EtG cut-off to retain the sensitivity of the cut-off, making analysis even more demanding. In some cases, a maximum sensitivity for DoA and BZD-Z might be required, and cut-off will not be applied. In such a case it could be reasonable to sample from the periphery of the scalp to increase the chance of detecting the analytes. If this approach is used, the interpretation should take into account that the metabolite-parent ratios could be lower. In the authors' opinion, the periphery of the haircut and especially the forehead might more likely be externally contaminated than the hair at the vertex. Therefore, this should be investigated before we can fully recommend such an approach.
In summary, hair samples should be taken from the vertex posterior and directly adjacent to each other. The location of sampling should be documented, especially if sampling was done in a different region or the strands were not taken adjacent to each other. If strands from non-vertex regions are used, the interpretation of the results should take this into consideration and the results should be interpreted carefully by an expert in this field.

4.6. Limitations of the Projects and Applied Methodologies

Even though the projects were very successful, as always there would have been some room for improvement. The major limitations of project 1 are clearly that it was a one-person study and EtG was the only investigated forensically relevant substance. These limitations were fully addressed in project 3. Project 1 did not address the pathways for incorporation, but project 3 did partially address this limitation as well. The method for caffeine measurement was not fully validated. However, the most important parameters were assed and the exact values in the hair are not important, so this is only a minor limitation.

In project 3 there were several limitations. Firstly, sebum as a possible incorporation pathway was not investigated. While there are a number of possible ways to collect sebum such as Sebutape® or cigarette paper,[110] the amount of sebum is hard to quantify as sebum cannot be collected without also collecting sweat, and the quantification of sebum amounts often requires specialized instruments such as Sebumeter® or Glossymeter®. Also the excretion rate of sebum is rather low, so collection would have had to be done over a longer time period.[111] As this was not practical for the study, the sebum excretion rates were not measured.
Secondly, a direct correlation of the sweat rates with the concentration data was not possible. This was not possible as the size and orientation of the sweat pads were different than the

sampled hair areas. Also, the exact sweat pad locations on the head in regard to the hair sample areas were not perfectly described. Therefore, only a visual comparison of the distributions could be done. In retrospect, it would have been beneficial to make more photos or videos of the head after sampling and during sweat collection. The photos could have been made in a way to allow the creation of a 3D-model. With the 3D-model, exact locations of sweat pads and sample areas could be overlaid and some calculations would have been possible. Still, from the visual assessment it is apparent that the sweating rates show a similar pattern as the hair concentrations of most DoA and BZD-Z. The method for sweat measurement was not validated. However, similar methods using sweat pads made of the same material have been validated.[112] The only notable change is the arrangement of many sweat pads on a cycling cap.

Thirdly, the head skin perfusion measurement with laser Doppler anemometry suffers from a few limitations. Pre-experiments had shown that the hair stubbles that remain after cutting the hair negatively influenced the results of the laser Doppler measurement. To eliminate this factor, the heads of the participants were shaved after cutting the hair. While this improved the measurement, the shaving process sometimes caused some skin rashes. Theses rashes showed much higher perfusion rates than the surrounding skin. A close visual inspection of the head was done, and photographic notes of any skin damage were made. These areas were not included in the perfusion measurements. It is likely that occasionally some damage was not seen which could cause perfusion rates to be too high in a few areas. A more crucial limitation is that the penetration depth of the used laser is about 0.3-0.4 mm.[113] The perfusion in the skin is supplied via two horizontal layers of blood vessels, the upper subpapillary plexus and the lower dermal plexus.[25] The hair roots are supplied via the dermal plexus. The perfusion measurement likely mainly measured the blood flow in the subpapillary plexus. The two layers are connected via many vertical blood vessels, and we assume that the perfusion in one layer is representative of the other. However, the perfusion results should be interpreted with caution. Other methods to measure the perfusion were considered. A lab focusing on optical coherence tomography,[114] which is regularly applied in ocular biometry, and has been used to image the microcirculation in mice ears and even of human skin in vivo[115,116] was contacted to see if this method could be applied to image the scalp microcirculation. Unfortunately, the methodology proved to be unsuitable for scalp skin. Using MRI measurements for imaging the microcirculation was considered, but the use of contrast agents was not an acceptable option and the resolution would have been an issue. Therefore, we decided to use the laser-Doppler imaging technique.

Fourthly, no female participants were enlisted. This was expected, as DoA consumption is more common in men than in women in Switzerland and men were expected to be more willing to part with their hair.

Fifthly, the self-reported consumption data is probably not very reliable. Participants were asked about their alcohol, DoA and BZD-Z consumption in a non-influencing way to avoid guiding their answers. However, many of the substances that were detected in the hair had not been reported. Often, no exact amounts for the drug consumption could be reported. Therefore, it was not attempted to correlate self-reported doses with hair concentrations.

As project 4 is still underway, it is too early to discuss limitations in detail, yet some limitations are immediately apparent. The study sets out to “realistically” contaminate the hair

with sweat containing EtG, DoA and BZD-Z. While definitely using more realistic conditions for incorporation via sweat than other conducted studies focusing on external contamination, it is hard to say what realistic actually means. The amount a person sweats, how often this person washes their hair, and countless other factors such as hair porosity, hair damage, hair length, etc. could be potential factors influencing the incorporation. Ultimately, the only time truly realistic conditions are certain, is in real life. In vivo studies have their own problems such as doing controlled studies, reproducibility, etc. Another limitation is uncertainty about the expected concentrations in the sweat. The concentrations of DoA/BZD-Z in the artificial sweat solution used in the study are estimated very roughly based on studies in which sweat patches were applied for abstinence testing or after controlled administration. Unfortunately, the amount of sweat that was secreted to produce the concentrations was not measured, so the concentrations in the sweat cannot be calculated. Instead, average sweat secretion rates were assumed to estimate the sweat concentrations. This is of course a large source of uncertainty for this study which is why a large concentration range was chosen for the low, mid, and high concentration levels. Another reason for this wide concentration range is that the consumed amounts of drugs also vary widely between consumers. Even with these limitations, we believe that by "realistically" modeling exposure and sweat concentrations, the study will provide valuable insights regarding to what extent substances can enter the hair through the sweat and if sweat rate differences are a possible explanation for the observed concentration differences across the scalp.

The major limitation of the Kratom project is of course that it is a one-person study. However, the study provides a valuable reference value for mitragynine concentrations in hair of a daily consumer, especially as the study participant reported very regular consumption of 3g/d. A limitation of the used methodology is that the LOD and LOQ of 7-Hy-Mitra were rather high. This might be the reason that no 7-Hy-Mitra was detected in any hair sample of the daily consumer. The LC-method as well as the ion source parameters could not be optimized for 7-Hy-Mitra, as the analytes were integrated into an existing DoA/BZD-Z method. Maybe some sensitivity gain would have been possible by optimizing all parameters for 7-Hy-Mitra. Mitragynine and 7-Hy-Mitra were chosen as they are the main psychoactive substances in Kratom. However, there are many more alkaloids in Kratom such as e.g. paynantheine, speciogynine, or speciociliatine, and some of these might have been better choices for detecting Kratom consumption. This would have to be further investigated if Kratom turns out to be a substance that needs to be regularly monitored.

The key limitation of the CBD study is that it is a one-person study. This was done as some information on THC blood levels after smoking CBD joints was needed quickly, as judging from the availability of CBD products in Swiss stores, CBD consumption is wide spread in Switzerland. As it is a one-person study, the results should of course be treated cautiously. Large intra-individual differences can be expected, especially considering that smoking is not a very standardized procedure. Studies with many participants, with longer application times, with more standardized methodologies, and with actual measurement of impairment are needed to judge the effects of CBD cannabis on driving capacity and fitness. Such a study has recently commenced at our institute. An additional limitation of the study was insufficient blood sampling frequency at the beginning of the experiment. Therefore, it is likely that the

true THC and CBD maxima in blood were missed. While this is not very important for this study, it would be good to characterize the pharmacokinetics more thoroughly in future studies.

4.7. Conclusion and Outlook

In summary, sampling location dependent hair concentration differences were thoroughly characterized for EtG, DoA, and BZD-Z for a collection of study participants using a newly developed method for the combined sample preparation of hair samples for the measurement of these substance classes. Large differences were observed with higher concentrations of most DoA and BZD-Z on the periphery of the haircut. EtG showed a reverse behavior to cocaine and the highest concentrations were found in the vertex region. The head skin perfusion rates and the head sweat rates were also characterized and compared with the concentration distribution patterns. From this comparison, we suspect differences in sweating rates to be largely responsible for the observed concentration differences. Perfusion differences likely only play a minor role. The plausibility of sweating as the major source of the concentrations differences is currently being investigated in a soaking experiment. As a side projects, a method for the measurement for mitragynine and 7-Hy-Mitra in hair was developed and was successfully applied to the hair of a regular Kratom user. The method was also applied to 300 routine samples which all tested negative for mitragynine. The CBD cannabis smoking study showed that after smoking a CBD joint, THC levels could rise above the legal cut-off concentration in blood for driving in Switzerland.
The results of the concentration distribution studies are important as they can influence recommendations for hair sampling. Additionally, the results can rationalize if large concentrations differences are found in a confirmation analysis. These concentration differences can regularly lead to hair results being interpreted differently when applying cut-offs depending on hair sampling location. It is not fair that a person should be judged differently depending on where a hair sample is taken. Hair samples should be taken at the vertex posterior as the recommended cut-off values by e.g. the SoHT are defined based on measurements from this area. It is unfortunately often not possible to sample hair from the vertex posterior due to e.g. male pattern baldness. These cases needed to be carefully interpreted. In the future, it should be discussed if applying the recommended cut-off values to hair taken from a different head region should be allowed at all, or if head region specific cut-offs need to be defined.

5. Literature

1. Cooper GA. Hair testing is taking root. *Ann Clin Biochem.* 2011;48(Pt 6):516-530.
2. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta.* 2006;370(1-2):17-49.
3. Kintz P. *Analytical and Practical Aspects of Drug Testing in Hair.* 1 ed: CRC Press; 2007.
4. Kintz P, Salomone A, Vincenti M. *Hair Analysis in Clinical and Forensic Toxicology.* 1 ed: Academic Press; 2015.
5. Sachs H. History of hair analysis. *Forensic Science International.* 1997;84(1):7-16.
6. Baumgartner AM, Jones PF, Baumgartner WA, Black CT. Radioimmunoassay of hair for determining opiate-abuse histories. *J Nucl Med.* 1979;20(7):748-752.
7. Kintz P. Drugs in Hair. In: Houck M, ed. *Forensic Toxicology*. London: Elsevier; 2018:223-227.
8. Buffoli B, Rinaldi F, Labanca M, et al. The human hair: from anatomy to physiology. *International Journal of Dermatology.* 2014;53(3):331-341.
9. Harkey MR. Anatomy and physiology of hair. *Forensic Sci Int.* 1993;63(1-3):9-18.
10. Jones LN. Hair structure anatomy and comparative anatomy11Dedicated to the memory of the late Dr. R. E. Rivett whose untimely death prevented his role in co-authorship. *Clinics in Dermatology.* 2001;19(2):95-103.
11. Gerace E, Veronesi A, Martra G, Salomone A, Vincenti M. Study of cocaine incorporation in hair damaged by cosmetic treatments. *Forensic Chemistry.* 2017;3:69-73.
12. Kaliyadan F, Gosai BB, Al Melhim WN, et al. Scanning Electron Microscopy Study of Hair Shaft Damage Secondary to Cosmetic Treatments of the Hair. *International Journal of Trichology.* 2016;8(2):94-98.
13. Shimomura Y, Ito M. Human Hair Keratin-Associated Proteins. *Journal of Investigative Dermatology Symposium Proceedings.* 2005;10(3):230-233.
14. Sperling LC. Hair anatomy for the clinician. *Journal of the American Academy of Dermatology.* 1991;25(1, Part 1):1-17.
15. Nakahara Y, Shimamine M, Takahashi K. Hair Analysis for Drugs of Abuse. III. Movement and Stability of Methoxyphenamine (As a Model Compound of Methamphetamine) along Hair Shaft with Hair Growth. *Journal of Analytical Toxicology.* 1992;16(4):253-257.
16. Paus R. Principles of hair cycle control. *The Journal of dermatology.* 1998;25(12):793-802.
17. Society of Hair Testing. Recommendations for hair testing in forensic cases. *Forensic Sci Int.* 2004;145(2-3):83-84.
18. Giovanoli-Jakubczak T, Berg GG. Measurement of Mercury in Human Hair. *Archives of Environmental Health: An International Journal.* 1974;28(3):139-144.
19. Randall VA. Androgens and hair growth. *Dermatologic Therapy.* 2008;21(5):314-328.
20. Loussouarn G. African hair growth parameters. *British Journal of Dermatology.* 2001;145(2):294-297.
21. Loussouarn G, Lozano I, Panhard S, Collaudin C, El Rawadi C, Genain G. Diversity in human hair growth, diameter, colour and shape. An in vivo study on young adults from 24 different ethnic groups observed in the five continents. *European journal of dermatology : EJD.* 2016;26(2):144-154.
22. Loussouarn G, El Rawadi C, Genain G. Diversity of hair growth profiles. *International Journal of Dermatology.* 2005;44(s1):6-9.
23. Randall VA, Ebling FJG. Seasonal changes in human hair growth. *British Journal of Dermatology.* 1991;124(2):146-151.
24. McGrath JA, Uitto J. Anatomy and Organization of Human Skin. *Rook's Textbook of Dermatology*: John Wiley and Sons; 2008.
25. Braverman IM. The cutaneous microcirculation. *J Investig Dermatol Symp Proc.* 2000;5(1):3-9.

26. Imanishi N, Kishi K, Chang H, Nakajima H, Aiso S. Three-dimensional venous anatomy of the dermis observed using stereography. *Journal of anatomy.* 2008;212(5):669-673.
27. Sonner Z, Wilder E, Heikenfeld J, et al. The microfluidics of the eccrine sweat gland, including biomarker partitioning, transport, and biosensing implications. *Biomicrofluidics.* 2015;9(3):031301.
28. Pappas A. Sebum and Sebaceous Lipids. In: Zouboulis CC, Katsambas AD, Kligman AM, eds. *Pathogenesis and Treatment of Acne and Rosacea*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014:33-41.
29. Downing DT, Strauss JS, Ramasastry P, Abel M, Lees CW, Pochi PE. Measurement of the Time Between Synthesis and Surface Excretion of Sebaceous Lipids in Sheep and Man. *Journal of Investigative Dermatology.* 1975;64(4):215-219.
30. Henderson GL. Mechanisms of drug incorporation into hair. *Forensic Science International.* 1993;63(1):19-29.
31. Pötsch L. A discourse on human hair fibers and reflections on the conservation of drug molecules. *International Journal of Legal Medicine.* 1996;108(6):285-293.
32. Shima N, Sasaki K, Kamata T, et al. Incorporation of zolpidem into hair and its distribution after a single administration. *Drug Metabolism and Disposition.* 2016.
33. Schrader J, Rothe M, Pragst F. Ethyl glucuronide concentrations in beard hair after a single alcohol dose: evidence for incorporation in hair root. *Int J Legal Med.* 2012;126(5):791-799.
34. Shima N, Nitta A, Kamata T, et al. Incorporation of zolpidem and methoxyphenamine into white hair strands after single administrations: Influence of hair pigmentation on drug incorporation. *Forensic Science International.* 2019.
35. Appenzeller BM, Schuman M, Yegles M, Wennig R. Ethyl glucuronide concentration in hair is not influenced by pigmentation. *Alcohol Alcohol.* 2007;42(4):326-327.
36. Rothe M, Pragst F, Thor S, Hunger J. Effect of pigmentation on the drug deposition in hair of grey-haired subjects. *Forensic Sci Int.* 1997;84(1-3):53-60.
37. Kelly RC, Mieczkowski T, Sweeney SA, Bourland JA. Hair analysis for drugs of abuse. Hair color and race differentials or systematic differences in drug preferences? *Forensic Sci Int.* 2000;107(1-3):63-86.
38. Rollins DE, Wilkins DG, Krueger GG, et al. The Effect of Hair Color on the Incorporation of Codeine into Human Hair. *Journal of Analytical Toxicology.* 2003;27(8):545-551.
39. Kronstrand R, Förstberg-Peterson S, KÅgedal B, Ahlner J, Larson G. Codeine Concentration in Hair after Oral Administration Is Dependent on Melanin Content. *Clinical Chemistry.* 1999;45(9):1485-1494.
40. Mieczkowski T. Assessing the potential for racial bias in hair analysis for cocaine: examining the relative risk of positive outcomes when comparing urine samples to hair samples. *Forensic Sci Int.* 2011;206(1-3):29-34.
41. Mieczkowski T, Newel R. An evaluation of patterns of racial bias in hair assays for cocaine: black and white arrestees compared. *Forensic Sci Int.* 1993;63(1-3):85-98.
42. Nakahara Y, Takahashi K, Kikura R. Hair Analysis for Drugs of Abuse. X. Effect of Physicochemical Properties of Drugs on the Incorporation Rates into Hair. *Biological & Pharmaceutical Bulletin.* 1995;18(9):1223-1227.
43. Robbins CR. *Chemical and Physical Behavior of Human Hair.* Springer Verlag, Berlin; 1994.
44. Barnes AJ, Smith ML, Kacinko SL, et al. Excretion of Methamphetamine and Amphetamine in Human Sweat Following Controlled Oral Methamphetamine Administration. *Clinical Chemistry.* 2008;54(1):172-180.
45. Caplan YH, Goldberger BA. Alternative specimens for workplace drug testing. *J Anal Toxicol.* 2001;25(5):396-399.
46. Concheiro M, Shakleya DM, Huestis MA. Simultaneous analysis of buprenorphine, methadone, cocaine, opiates and nicotine metabolites in sweat by liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem.* 2011;400(1):69-78.
47. Cone EJ, Hillsgrove MJ, Jenkins AJ, Keenan RM, Darwin WD. Sweat testing for heroin, cocaine, and metabolites. *J Anal Toxicol.* 1994;18(6):298-305.

48. Gambelunghe C, Rossi R, Aroni K, et al. Sweat Testing to Monitor Drug Exposure. *Annals of Clinical & Laboratory Science.* 2013;43(1):22-30.
49. Huestis MA, Cone EJ, Wong CJ, Umbricht A, Preston KL. Monitoring Opiate Use in Substance Abuse Treatment Patients With Sweat and Urine Drug Testing. *Journal of Analytical Toxicology.* 2000;24(7):509-521.
50. Huestis MA, Oyler JM, Cone EJ, Wstadik AT, Schoendorfer D, Joseph RE. Sweat testing for cocaine, codeine and metabolites by gas chromatography–mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications.* 1999;733(1):247-264.
51. Kacinko SL, Barnes AJ, Schwilke EW, Cone EJ, Moolchan ET, Huestis MA. Disposition of cocaine and its metabolites in human sweat after controlled cocaine administration. *Clin Chem.* 2005;51(11):2085-2094.
52. Kintz P, Brenneisen R, Bundeli P, Mangin P. Sweat testing for heroin and metabolites in a heroin maintenance program. *Clin Chem.* 1997;43(5):736-739.
53. Kintz P, Tracqui A, Mangin P, Edel Y. Sweat testing in opioid users with a sweat patch. *J Anal Toxicol.* 1996;20(6):393-397.
54. Levisky JA, Bowerman DL, Jenkins WW, Karch SB. Drug deposition in adipose tissue and skin: evidence for an alternative source of positive sweat patch tests. *Forensic Science International.* 2000;110(1):35-46.
55. Liberty HJ, Johnson BD, Fortner N. Detecting Cocaine Use Through Sweat Testing: Multilevel Modeling of Sweat Patch Length-of-Wear Data. *Journal of analytical toxicology.* 2004;28(8):667-673.
56. Pichini S, Navarro M, Pacifici R, et al. Usefulness of sweat testing for the detection of MDMA after a single-dose administration. *J Anal Toxicol.* 2003;27(5):294-303.
57. Schummer C, Appenzeller BM, Wennig R. Quantitative determination of ethyl glucuronide in sweat. *Ther Drug Monit.* 2008;30(4):536-539.
58. Schwilke EW, Barnes AJ, Kacinko SL, Cone EJ, Moolchan ET, Huestis MA. Opioid Disposition in Human Sweat after Controlled Oral Codeine Administration. *Clinical Chemistry.* 2006;52(8):1539-1545.
59. LeBeau MA, Montgomery MA, Brewer JD. The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. *Forensic Sci Int.* 2011;210(1-3):110-116.
60. Sachs H. Theoretical limits of the evaluation of drug concentrations in hair due to irregular hair growth. *Forensic Science International.* 1995;70(1):53-61.
61. Skopp G, Pötsch L, Mauden M. Stability of Cannabinoids in Hair Samples Exposed to Sunlight. *Clinical Chemistry.* 2000;46(11):1846-1848.
62. Favretto D, Tucci M, Monaldi A, Ferrara SD, Miolo G. A study on photodegradation of methadone, EDDP, and other drugs of abuse in hair exposed to controlled UVB radiation. *Drug Testing and Analysis.* 2014;6(S1):78-84.
63. Miolo G, Stocchero G, Vogliardi S, et al. A Study on Photostability of Ethyl Glucuronide in Hair Irradiated under Artificial Sunlight. *J Anal Toxicol.* 2019.
64. Agius R, Ferreira LM, Yegles M. Can ethyl glucuronide in hair be determined only in 3 cm hair strands? *Forensic Sci Int.* 2012;218(1-3):3-9.
65. Binz TM, Baumgartner MR, Kraemer T. The influence of cleansing shampoos on ethyl glucuronide concentration in hair analyzed with an optimized and validated LC-MS/MS method. *Forensic Sci Int.* 2014;244:20-24.
66. Luginbuhl M, Nussbaumer S, Weinmann W. Decrease of ethyl glucuronide concentrations in hair after exposure to chlorinated swimming pool water. *Drug Test Anal.* 2018;10(4):689-693.
67. Tsanaclis L, Kingston R, Wicks J. Testing for alcohol use in hair: is ethyl glucuronide (EtG) stable in hair? *Ann Toxicol Anal.* 2009;21(2):67-71.
68. Agius R. Utility of coloured hair for the detection of drugs and alcohol. *Drug Testing and Analysis.* 2014;6(S1):110-119.
69. Jurado C, Kintz P, Menendez M, Repetto M. Influence of the cosmetic treatment of hair on drug testing. *Int J Legal Med.* 1997;110(3):159-163.

70. Martins Ferreira L, Binz T, Yegles M. The influence of ethanol containing cosmetics on ethyl glucuronide concentration in hair. *Forensic Sci Int.* 2012;218(1-3):123-125.
71. Petzel-Witt S, Pogoda W, Wunder C, Paulke A, Schubert-Zsilavecz M, Toennes SW. Influence of bleaching and coloring on ethyl glucuronide content in human hair. *Drug Test Anal.* 2018;10(1):177-183.
72. Suesse S, Pragst F, Mieczkowski T, et al. Practical experiences in application of hair fatty acid ethyl esters and ethyl glucuronide for detection of chronic alcohol abuse in forensic cases. *Forensic Sci Int.* 2012;218(1-3):82-91.
73. Morini L, Zucchella A, Polettini A, Politi L, Groppi A. Effect of bleaching on ethyl glucuronide in hair: An in vitro experiment. *Forensic Science International.* 2010;198(1):23-27.
74. Hartwig S, Auwärter V, Pragst F. Effect of hair care and hair cosmetics on the concentrations of fatty acid ethyl esters in hair as markers of chronically elevated alcohol consumption. *Forensic Science International.* 2003;131(2):90-97.
75. Li J, Xie Q, Gao W, et al. Time course of cortisol loss in hair segments under immersion in hot water. *Clinica Chimica Acta.* 2012;413(3):434-440.
76. Society of Hair Testing. Consensus for the Use of Alcohol Markers in Hair for Assessment of both Abstinence and Chronic Excessive Alcohol Consumption. 2016; http://www.soht.org/index.php/consensus. Accessed 17.11.2016, 2016.
77. Romano G, Barbera N, Lombardo I. Hair testing for drugs of abuse: evaluation of external cocaine contamination and risk of false positives. *Forensic Science International.* 2001;123(2):119-129.
78. Moosmann B, Roth N, Auwarter V. Hair analysis for Delta(9) -tetrahydrocannabinolic acid A (THCA-A) and Delta(9) -tetrahydrocannabinol (THC) after handling cannabis plant material. *Drug Test Anal.* 2016;8(1):128-132.
79. Hoelzle C, Scheufler F, Uhl M, Sachs H, Thieme D. Application of discriminant analysis to differentiate between incorporation of cocaine and its congeners into hair and contamination. *Forensic Sci Int.* 2008;176(1):13-18.
80. Mieczkowski T. Distinguishing passive contamination from active cocaine consumption: assessing the occupational exposure of narcotics officers to cocaine. *Forensic Science International.* 1997;84(1):87-111.
81. Villain M, Muller J-F, Kintz P. Heroin markers in hair of a narcotic police officer: Active or passive exposure? *Forensic Science International.* 2010;196(1):128-129.
82. Pichini S, Garcia-Algar O, Alvarez AT, et al. Pediatric exposure to drugs of abuse by hair testing: monitoring 15 years of evolution in Spain. *Int J Environ Res Public Health.* 2014;11(8):8267-8275.
83. Pragst F, Krumbiegel F, Thurmann D, et al. Hair analysis of more than 140 families with drug consuming parents. Comparison between hair results from adults and their children. *Forensic Sci Int.* 2019;297:161-170.
84. Moosmann B, Roth N, Hastedt M, Jacobsen-Bauer A, Pragst F, Auwärter V. Cannabinoid findings in children hair – what do they really tell us? An assessment in the light of three different analytical methods with focus on interpretation of Δ9-tetrahydrocannabinolic acid A concentrations. *Drug Testing and Analysis.* 2015;7(5):349-357.
85. Cairns T, Hill V, Schaffer M, Thistle W. Removing and identifying drug contamination in the analysis of human hair. *Forensic Science International.* 2004;145(2):97-108.
86. Schaffer M, Hill V, Cairns T. Hair Analysis for Cocaine: The Requirement for Effective Wash Procedures and Effects of Drug Concentration and Hair Porosity in Contamination and Decontamination. *Journal of Analytical Toxicology.* 2005;29(5):319-326.
87. Stout PR, Ropero-Miller JD, Baylor MR, Mitchell JM. External contamination of hair with cocaine: evaluation of external cocaine contamination and development of performance-testing materials. *J Anal Toxicol.* 2006;30(8):490-500.
88. Tsanaclis L, Nutt J, Bagley K, Bevan S, Wicks J. Differentiation between consumption and external contamination when testing for cocaine and cannabis in hair samples. *Drug Test Anal.* 2014;6 Suppl 1(S1):37-41.

89. Morris-Kukoski CL, Montgomery MA, Hammer RL. Analysis of extensively washed hair from cocaine users and drug chemists to establish new reporting criteria. *J Anal Toxicol.* 2014;38(9):628-636.
90. Cuypers E, Flanagan RJ. The interpretation of hair analysis for drugs and drug metabolites. *Clin Toxicol (Phila).* 2018;56(2):90-100.
91. Han E, Park Y, Yang W, et al. The study of metabolite-to-parent drug ratios of methamphetamine and methylenedioxymethamphetamine in hair. *Forensic Sci Int.* 2006;161(2-3):124-129.
92. Madry MM, Rust KY, Guglielmello R, Baumgartner MR, Kraemer T. Metabolite to parent drug concentration ratios in hair for the differentiation of tramadol intake from external contamination and passive exposure. *Forensic Sci Int.* 2012;223(1-3):330-334.
93. Gambelunghe C, Rossi R, Aroni K, et al. Norcocaine and cocaethylene distribution patterns in hair samples from light, moderate, and heavy cocaine users. *Drug Testing and Analysis.* 2017;9(2):161-167.
94. Minoli M, Casati S, Angeli I, et al. Analysis of hydroxy-cocaine metabolites as evidence of cocaine consumption: Identification by parent ion search and quantitation by UHPLC-MS/MS in hair. *J Pharm Biomed Anal.* 2019;172:167-174.
95. Schaffer M, Cheng CC, Chao O, Hill V, Matsui P. Analysis of cocaine and metabolites in hair: validation and application of measurement of hydroxycocaine metabolites as evidence of cocaine ingestion. *Anal Bioanal Chem.* 2016;408(8):2043-2054.
96. Musshoff F, Thieme D, Schwarz G, Sachs H, Skopp G, Franz T. Determination of hydroxy metabolites of cocaine in hair samples for proof of consumption. *Drug Test Anal.* 2018;10(4):681-688.
97. Mangin P, Kintz P. Variability of opiates concentrations in human hair according to their anatomical origin: head, axillary and pubic regions. *Forensic Science International.* 1993;63(1):77-83.
98. Hartwig S, Auwärter V, Pragst F. FATTY ACID ETHYL ESTERS IN SCALP, PUBIC, AXILLARY, BEARD AND BODY HAIR AS MARKERS FOR ALCOHOL MISUSE. *Alcohol and Alcoholism.* 2003;38(2):163-167.
99. Pianta A, Liniger B, Baumgartner MR. Ethyl Glucuronide in Scalp and Non-head Hair: An Intra-individual Comparison. *Alcohol and Alcoholism.* 2013;48(3):295-302.
100. Kerekes I, Yegles M, Grimm U, Wennig R. Ethyl glucuronide determination: head hair versus non-head hair. *Alcohol Alcohol.* 2009;44(1):62-66.
101. Dussy F, Carson N, Hangartner S, Briellmann T. Is one hair lock really representative? *Drug Test Anal.* 2014;6 Suppl 1:5-8.
102. Cooper GA, Kronstrand R, Kintz P, Society of Hair T. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int.* 2012;218(1-3):20-24.
103. Salomone A, Tsanaclis L, Agius R, Kintz P, Baumgartner MR. European guidelines for workplace drug and alcohol testing in hair. *Drug Testing and Analysis* 2016;8(10):996-1004.
104. Ropero-Miller JD, Huestis MA, Stout PR. Cocaine analytes in human hair: evaluation of concentration ratios in different cocaine sources, drug-user populations and surface-contaminated specimens. *J Anal Toxicol.* 2012;36(6):390-398.
105. Cuypers E, Flinders B, Boone CM, et al. Consequences of Decontamination Procedures in Forensic Hair Analysis Using Metal-Assisted Secondary Ion Mass Spectrometry Analysis. *Analytical Chemistry.* 2016;88(6):3091-3097.
106. SoHT. 2019 Consensus for the use of alcohol markers in hair for supporting the assessment of abstinence and chronic alcohol consumption. 2019; https://www.soht.org/images/pdf/Revision_2019_Alcoholmarkers.pdf. Accessed 08.08.2019, 2019.
107. Lees R, Kingston R, Williams TM, Henderson G, Lingford-Hughes A, Hickman M. Comparison of ethyl glucuronide in hair with self-reported alcohol consumption. *Alcohol Alcohol.* 2012;47(3):267-272.

108. Kronstrand R, Brinkhagen L, Nystrom FH. Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months. *Forensic Sci Int.* 2012;215(1-3):51-55.
109. Crunelle CL, Yegles M, De Doncker M, et al. Hair ethyl glucuronide concentrations in teetotalers: Should we re-evaluate the lower cut-off? *Forensic Sci Int.* 2017;274:107-108.
110. Joseph JRE, Oyler JM, Wstadik AT, Ohuoha C, Cone EJ. Drug Testing with Alternative Matrices I. Pharmacological Effects and Disposition of Cocaine and Codeine in Plasma, Sebum, and Stratum Corneum. *Journal of Analytical Toxicology.* 1998;22(1):6-17.
111. Downing DT, Stewart ME, Strauss JS. Estimation of Sebum Production Rates in Man by Measurement of the Squalene Content of Skin Biopsies. *Journal of Investigative Dermatology.* 1981;77(4):358-360.
112. Morris NB, Cramer MN, Hodder SG, Havenith G, Jay O. A comparison between the technical absorbent and ventilated capsule methods for measuring local sweat rate. *J Appl Physiol (1985).* 2013;114(6):816-823.
113. Fredriksson I, Larsson M, Stromberg T. Measurement depth and volume in laser Doppler flowmetry. *Microvascular research.* 2009;78(1):4-13.
114. Fercher AF. Optical coherence tomography - development, principles, applications. *Z Med Phys.* 2010;20(4):251-276.
115. Choi WJ, Reif R, Yousefi S, Wang RK. Improved microcirculation imaging of human skin in vivo using optical microangiography with a correlation mapping mask. *J Biomed Opt.* 2014;19(3):36010.
116. Reif R, Wang RK. Label-free imaging of blood vessel morphology with capillary resolution using optical microangiography. *Quant Imaging Med Surg.* 2012;2(3):207-212.