

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## New Challenges and Perspectives in Hair Analysis

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1567335> since 2016-06-20T10:13:12Z

*Publisher:*

Academic Press

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

## 12. New Challenges and Perspectives in Hair Analysis

**Marco Vincenti and Pascal Kintz**

### **Abstract**

During the last decade, hair analysis has considerably strengthened its role and reputation as a valuable investigation tool to provide crucial information about the chronological exposure of controlled subjects to targeted chemicals during extended periods of time. Consequently, hair analysis is nowadays accepted as the foremost evidence of biological exposure in a variety of legal inquiries, particularly in the field of forensic toxicology. With the growing role, also the responsibility taken by the toxicologists to correctly interpret the results of hair analysis has increased in proportion, demanding in-depth study on all the mechanisms by which exogenous substances are incorporated inside the keratin structure and eventually released.

The closing chapter of the present book gives credit to the ongoing efforts that the scientific community is making to balance the rapidly expanding investigation opportunities offered by the technological advancements of analytical instrumentation with the need to reinforce the knowledge about the conditioning factors and the sources of uncertainty that ultimately determine the analytical outcome of hair analysis. The most important among of these issues are considered in dedicated chapters of the present book; other influencing factors of emerging importance are listed and portrayed, with reference to the existing literature and its upcoming extension.

In particular, the following potential sources of qualitative, quantitative or chronological bias in hair analysis results are illustrated: (i) the damage produced to the hair structure and its constituents by physical and chemical agents; (ii) the hair color and its melanin content; (iii) the dependence of target analytes distributions along the hair shaft from the prevalent way of their excretion; (iv) individual variability ascribed to genetic, personal, physio- pathological, and behavioral factors.

The second part of the chapter is dedicated to the most striking innovations recently arose in hair analysis and their prospective evolution in the forthcoming years. Specific attention is devoted to (i) untargeted and broad-spectrum toxicological analysis, (ii) the determination of minor drug metabolites and biomarkers; (iii) the analytical discrimination of enantiomers; (iv) fine-segmental and single hair analysis. The dependence of the potential evolution of this forefront research on the progresses of chromatographic and mass spectrometric instrumentation, together with their gradual availability, is underlined with reference to the most recent technological advancements.

## **Keywords**

Hair analysis; cosmetic treatment; hair porosity; hair color; melanin; factors of bias; segmental hair analysis; single hair analysis; drug metabolite; drug biomarker; alcohol biomarker; enantiomer discrimination; MALDI imaging; hair imaging; LC-MS/MS.

### **12.1. Introduction**

The relatively short history of hair analysis has gradually evolved from an early stage, when most research efforts were addressed to the discovery of the new opportunities offered by this unique memory-keeping biological matrix, to the recent consolidation stage, in which the search for innovative procedures and applications for hair analysis is balanced by a thoughtful reconsideration of unexplored variables that may influence the final analytical results. This two-pronged character of scientific research dedicated to hair analysis will most likely persist in the future, because the continuous innovation of instrumental technology offers unprecedented investigation opportunities but, on the other hand, the scientific community is requested to provide solid interpretation foundation to their analytical outcomes.

These two components of scientific research on hair analysis, respectively addressed to innovative and conservative issues, emblematically correspond to the counterparts of any lawsuit – prosecution and defense - and, under such circumstances, provide good reasoning elements for each one. Both sides of the coin offer challenging objectives to be pursued, which will represent the focus of most research struggles in the forthcoming years. These two perspective aspects are examined in the subsequent paragraphs.

### **12.2. Conditioning factors and sources of variability**

The two extreme steps of hair analysis are respectively (i) the intake of a certain substance by a subject and (ii) the quantitative determination of the same substance and/or its metabolites in the hair of this subject, collected after a delay of days/weeks/months from the intake. The practical success of hair testing in a variety of circumstances, together with its demonstrated trustworthiness in providing crucial information, does not conceal the fact that the final analytical result is conditioned by a large number of biological, chemical, and physical processes, which in turn depend on several causes, ranging from individual and environmental to methodological. Each process generates a source of variability, that must be taken into account when the analytical data have to be interpreted [1,2].

Table 1. Influencing factors and processes

- Mean of intake/administration (ingestion, injection, inhalation, absorption)
- Metabolism of the substance (kinetics & metabolites' distribution)
- Distribution of the marker into the body fluids (blood, sweat, sebum – plus urine, etc.)
- Mechanisms of marker transfer from blood, sweat, sebum to hair and relative importance
- Mechanism of incorporation of the marker into the keratin structure
- Interaction of the marker with melanin by hydrogen bonding (hair color)
- Longitudinal and radial diffusion of the marker
- Washing-out phenomena (porosity)
- External contamination
- Physical transformation processes (heat, light)
- Chemical transformation processes (oxidants, dyes, strong alkaline agents)
- Type of hair (head, pubic, axillary, chest, legs)
- Growing rate of hair and phases
- Site of sampling (for head hair)
- Length of hair sampled and investigated
- Decontamination procedure
- Hair fragmentation
- Extraction of the marker
- Purification of the extract
- Analytical method
- Quantification method

Table 2. Classification of influencing factors

- Individual:
  - Genetic determiners & polymorphisms (& their expression)
  - Personal factors (gender, age, body-mass index)
  - Physio-pathological factors
  - Behavioral factors:
    - Mode and frequency of substance intake
    - Hygienic habits
    - Cosmetic habits
    - Diet habits
    - Intake of interfering medicines
    - Clothing habitually used (fabric, heavy/light, special clothes, i.e. hat, foulard, scarf)
    - Other frequent sources of (self)-contamination (i.e. pillow, armchair with headrest)
- Environmental (domestic, work-related, leisure-related)
- Climatic (meteorological, geographical, seasonal)
- Methodological (sampling, sample treatment, analysis)

A possible sequence of steps and processes, occurring along the way that the designated substance (and/or its metabolites) may be subjected to, before it is detected at the final mass spectrometric instrumentation, is reported in Table 1. A classification of the factors that produce these processes, and the consequent variability of the expected results, is reported in Table 2. Quite

obviously, the variability associated with each of these factors varies from negligible to relevant, depending on the specific substances under investigation, and the population of subjects considered. Each factor could ideally be treated as a single element of contribution to the expanded uncertainty of hair analysis or, more practically, their clustered effect can be evaluated by holistic approaches.

In the past, the effect of various experimental aspects of hair analysis has been studied in detail [3,4], and the procedures developed to decontaminate, fragment, extract, purify, and analyze hair samples are extensively reviewed in a specific chapter of the present book. Also the physiology of hair growth and the mechanisms by which xenobiotic substances are transferred from body fluids to the hair surface and bulb, and then incorporated into the keratin structure, have been extensively investigated throughout the years. The remaining factors listed in Table 1 still represent subjects of controversial debate [5], [6,7] or either the discussion about their effect just started within the scientific community. These themes characterize a significant part of the recent research on hair analysis and will most likely constitute upcoming challenges for the future. A specific chapter of this book is devoted to the problem of external contamination, where the reader can find extensive review of the pertinent scientific literature.

A group of interrelated processes potentially undermines the permanence inside the hair structure of the incorporated compounds. Various chemical and physical phenomena may partially modify or remove the original substances from the hair, ultimately leading to biased results. These phenomena may produce the degradation of the analytes molecular structure due to the effects of heat, light, and strong oxidants, acids, and bases. Besides direct decomposition processes, the same agents may also cause the degradation of the keratin structure, leading to increased porosity of the hair surface, and facilitated release of the incorporated substances. The extent of these phenomena also depend on the chemical stability of the investigated compounds and the strength of their interactions with hair constituents, in particular melanin. Recent experimental studies have investigated both single factors and aggregated phenomena.

### ***12.2.1. Physical and chemical agents***

The effect of heating on ethyl glucuronide (EtG) concentration, consequent to the repeated application of a hair straightener under mild conditions, was investigated by in-vitro experiments [8]. Although significant changes were observed with respect to non-treated hair locks, opposite variations were recorded for each half of the investigated population, with a prominent dependence on hair color. Even for such a relatively simple experiment, at least two coexisting phenomena have to be recalled to explain the results, namely thermal degradation (for EtG drop) and more efficient

extraction from the keratin matrix (for EtG increase) [8]. On the other hand, EtG proved to be extremely stable within the keratin matrix over extended periods of time, as was clearly demonstrated by its determination on mummy hair samples, collected several hundred years after death [9].

Another physical effect that recently draw the attention was the potential action of the solar light [10], possibly playing a role in the degradation of hair-incorporated substances, especially during the summer season. Real positive hair samples were exposed to UV-B radiation [11] and the change of drug content (methadone, morphine, cocaine, and their metabolites) was recorded, revealing particularly extensive decomposition for methadone, and higher stability for drugs incorporated in thick dark hair. Further experimental work, using true solar light and in-vivo conditions, appears to be needed in the future, before a definite answer may be given about the potential transformation of hair content, induced by exposition to solar light.

While physical agents do not appear to produce substantial modification of the hair cuticle [12], considerable alteration of hair porosity [13] is induced by the chemical products utilized in several cosmetic treatments, including perms, bleaching, and dyeing [14–16]. Under these circumstances, it is virtually impossible to distinguish the release of the incorporated substances through the pores and cavities produced at the hair surface [12,15], from their possible loss by chemical reaction with cosmetic products. The earlier findings [14–16] on the effects of cosmetic treatments have been confirmed by the most recent literature, even if the risk of observing a large number of false-negative results from cosmetically-treated hair has been recently contradicted, on the basis of statistical criteria, founded on a large population of treated and non-treated authentic hair samples [17]. Particularly for alcohol abuse testing by hair EtG determination, the percentage of positive samples turned out to be the same for treated and non-treated hair, demonstrating the reliability of the EtG biomarker also under stressed conditions. Once more, this paper highlights the differences occurring between the studies conducted under real in-vivo conditions [17] and those performed by in-vitro experiments [18], which occasionally lead to opposite conclusions.

Recent studies carried out to investigate the effects of cosmetic treatments on hair content stability, have chiefly concerned amphetamines [13,19,20] and cocaine [12,13,21]. In general, the variability of hair features in humans (thickness, color, curly/straight), together with the vast assortment of cosmetic products, have prevented any systematic study of their effects, but rather spot conditions have been tested. For amphetamines, modest but statistically significant loss was recorded upon the single application of a bleaching agent (for 40 minutes) [19], or different dyes [20]. Slightly lower amphetamines release was also produced by repeated (10 times) washing with liquid soap, followed by UV irradiation (for 2 h) [20]. The effect of hydrogen peroxide on hair

containing cocaine, from both active user and external contamination, was recently investigated by MALDI-TOF imaging on single hairs [21]. It was deduced that extensive oxidation and removal of cocaine and its metabolites/oxidation products occurred in both cases, making the detectability of cocaine abuse from bleached hair problematic; the overall effect was attributed to a combination of the cleavage of melanin binding and direct oxidation processes [21]. However, a possibility was considered that cocaine removal could be partially promoted by treating previously cut hair, unlike in real cases.

Another important point evidenced by several studies, is that contamination from external sources is enhanced in highly porous hair, such as that previously subjected to repeated cosmetic treatments [12,15]. This increases the chance of producing false-positive results, whenever an unsuited decontamination policy is executed [13,22], taking into account that incorrect hair washing can itself produce significant hair damage [23].

A well-structured strategy has been recently devised [13] to measure the degree of hair porosity, to deduce the differences occurring between “normal” cosmetic treatments from “extreme” treatments – possibly carried out by drug users to circumvent positive hair testing -, to distinguish external contamination from actual intake for both normal and damaged hair, and to establish a correct washing/decontamination protocol for each of these cases. As previously cited [17], mild cosmetic treatments applied according to the vendor instructions appear to be compatible with hair testing for drug and alcohol abuse [13]. Likewise, the sporadic use of special cleansing shampoos, promoted as capable of “cleaning” the hair from drugs and alcohol biomarkers, was demonstrated not to affect EtG hair concentration [24], nor to remove a significant amount of any drug, including THC, cocaine, heroin, 6-MAM, morphine, codeine, methadone, and several amphetamines [25].

### ***12.2.2. Interaction between hair constituents and incorporated substances***

A fundamental aspect of hair analysis that has repercussions on a variety of observed phenomena is the nature of the bonds that the incorporated substances form with hair constituents, particularly melanin. These chemical interactions have been investigated in the past, mainly using in-vitro experiments [26–28], animal models [29–32], and statistical inference [26,33,34], but the increasing interest captured by hair analysis and its ever expanding applications will propel more systematic work on the basics of human hair capturing capability in the future.

Human hair contains essentially two types of melanin oligomers: eumelanin, comprising 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid units, and pheomelanin, incorporating

benzothiazine and benzothiazole units. Both have acidic properties, but the higher density of carboxylic groups confers to eumelanin a considerably stronger acidic character. Eumelanin is more abundant in black and brown hair, whereas pheomelanin predominates in blond and red hair. Detailed description of melanin structures and their binding properties may be found in a specific text [35]. On the basis of theoretical considerations and some experimental evidence, basic substances are expected to strongly bind to eumelanin via hydrogen bonds and charge-transfer interactions, allegedly resulting in biased outcomes, that depend on the hair color of the tested subject. On the other hand, acidic and neutral substances are supposed to produce only weak interaction with both types of melanin, yielding similar incorporation from hair of any color. For these substances, the structural hindrance provided by the compact keratin net of hair plays a substantial role in their entrapment and prevents their easy removal.

Among drugs of abuse, a direct comparison between basic amphetamine and cocaine, and their neutral or zwitterion counterparts, namely N-acetylamphetamine and benzoylecgonine, was conducted by measuring their in-vitro affinity toward various types of melanin [27]. It was confirmed that basic drugs are strongly bound by eumelanin-rich substrates by means of non-covalent multiple interactions with ionic character, whereas pure pheomelanin does not bind the same substances to any extent. Likewise, neutral N-acetylamphetamine and benzoylecgonine showed little or no interaction with any type of melanin, as they cannot establish highly polarized interaction with the substrates [27]. Some evidence of covalent binding between amphetamines and melanin was observed in experiments of melanin synthesis in the presence of amphetamine, namely under in-vitro conditions very distant from reality [28].

Animal models represent a practical way to bypass the ethical problem of administering illegal drugs to humans for experimental trials, but the extension to mankind of conclusions drawn from animal models is frequently questioned. An interesting recent study involved the administration of codeine and morphine to rats carrying white and dark grey spots of hair on their body. Although the two substances could be detected in both types of hair, the concentrations measured in dark grey hair was 8 to 40 times higher than in white hair [31]. An analogous approach was utilized to verify the possible influence of hair pigmentation on EtG [29] and FAEE [30] concentrations, after ethanol administration to eleven and six rats, respectively. Opposite conclusions with respect to opiates were drawn, since highly correlated and almost coincident results were obtained from pigmented and white hair, demonstrating that melanin does not bind EtG [29] nor FAEE [30]. Noteworthy, EtG is highly hydrophilic, whereas FAEE are highly hydrophobic, but both biomarkers have no basic properties nor positive charge, resulting in scarce interaction with melanin. Likewise, no influence of pigmentation was observed in the hair



distribution of the new cannabimimetic agent JWH-073 (and its metabolites), following its administration to five rats [32].

Some statistical studies were undertaken around the year 2000, where the positivity rate among different ethnic groups was compared from hair and urine testing for several drugs of abuse [26,33,34]. Mieczkowski and co-workers [33] recorded almost identical results from urine and hair testing, while modest differences of positivity rates from the expected values could be entirely attributed to preferences for specific drugs among ethnic groups. No hair color bias, nor selective binding of drugs to hair of a certain color, nor metabolic difference among ethnic groups was evidenced [33,34]. While it is admitted that color may play a role in the accumulation of drugs in hair, large within-group variations occurring in other determining factors nonetheless obscure any clear evidence of color bias [34]. Kidwell and co-workers argued that ethnic differences in drug hair-binding capacity do exist, but they are not due to the hair color (which is typically black for both Asian and African people), but rather to different hair permeability, hair care and personal hygiene habits, and also the route of drug administration or passive exposure [26]. They also argued that the “positivity rate” is not a valuable means of detecting differences, because it is largely dependent on the cut-off threshold used for rating a positive outcome. Indeed, while the high sensitivity of modern analytical instrumentation has overcome the problem of achieving sufficiently low detection thresholds, it has emphasized the need to discriminate between active intake of drugs from passive exposure. The problem of the correct choice of cut-off values was also posed in an in-vivo study, that compared the hair concentration of codeine for 44 subjects after repeated administration of the drug [36]. This study evidenced large differences in codeine hair-binding for groups with different hair color, and high correlation with melanin hair content, possibly resulting in biased positive vs. negative judgment, unless the results are corrected for melanin content [36,37].

The latter studies demonstrated the advantages of in-vivo experiments conducted on humans, with respect to model and statistical investigations, to provide direct answer and practical solutions to the interpretation queries that forensic toxicology consistently poses. Moreover, in-vivo studies provide easier control of variability sources and avoid any cruelty to animals, an issue of increasing concern. The application of safe experimental setting, ethical committee control, and clear informed consent policy has made in-vivo trials on humans gradually accepted, encouraging their use in the recent past and, most likely, in forthcoming years.

A controlled administration of codeine and cocaine to ten volunteers demonstrated that the concentrations in hair of codeine, cocaine, and their metabolites is dose-related and linearly correlated with total melanin content in the hair [38]. In contrast, routine analysis of 8,687 hair

samples, found positive to both cocaine and benzoylecgonine, showed no difference between brown and black hair samples for cocaine, and little difference for benzoylecgonine, with no practical consequences on the positivity rate [39].

The strategy to compare results obtained from hair of different color but arising from the same subject is appropriately applied to eliminate all the sources of inter-individual variability from the experimental data. Following this strategy, samples are collected from subjects with grey hair, then the white and pigmented hairs are separated, and processed independently [40]–[42]. Significantly higher concentrations were observed in pigmented hair, with respect to white hair, for cocaine, amphetamines [42], and several prescription drugs with basic properties, such as amitriptyline [41], chlorpromazine, and clozapine [40]. Highly dissimilar (1/100) hair incorporation of zolpidem, after single administration, was hypothetically attributed to ethnic differences – in terms of melanin content – between Asian and European volunteers [43,44]; indeed, largely different zolpidem concentrations were observed also between white and black hairs of a single subject [44]. In the same way, Appenzeller and co-workers collected samples from 21 deceased persons with grizzled hair, with which they convincingly demonstrated that the concentration of EtG in hair does not depend on its melanin content [45]. The actual incorporation of all these substances in white hair, not containing melanin, even though at lower concentration, together with the incorporation of substances that do not interact with melanin, such as EtG, prove that keratin and other hair proteins play an important role in the binding of drugs in hair [40].

The recent introduction of a huge number of new synthetic drugs into the illegal market has already opened the problem of their updated detection in hair samples [46]–[49], but leaves the investigation field of their interaction with hair components still unexplored. Large differences exist in the structure and chemical properties between mostly neutral cannabimimetic agents and basic phenethylamines and cathinones, but also among the substances of the same class, which are likely to be reflected into their binding to melanin and keratin. The study of these interactions by both theoretical and experimental approaches represents one of the emerging challenges for the upcoming toxicology research.

### ***12.2.3. Distribution of the xenobiotic substances within the hair***

Incorporation of drugs and other xenobiotic substances inside the hair structure occurs from several sources (blood, sweat, sebum) with different mechanisms, each contributing to the final hair composition, depending on the specific substance considered. The second variable that determines the substances distribution along the hair length is its growth rate and the succession of anagen,

catagen, and telogen phases. The third important parameter to be considered is the frequency of intake: single, occasional, variable in dose, or continuous. All these aspects are extensively reviewed in a specific chapter of the present book; although the hair inclusion mechanisms are still partially unknown, their fundamental principles have been studied with sufficient detail to constitute a solid foundation for drawing quite sophisticated interpretations.

However, other confounding factors are occasionally present under specific circumstances, that may modify the expected distribution of the substances under investigation within the hair. For example, while blood can release its constituents only inside the bulb, sweat and sebum might be dispersed along a larger portion of the hair shaft, from which diffuse uptake of xenobiotics could arise. Other body fluids, specifically urine, may contaminate hair, determining wide alteration of its composition, as is frequently observed for pubic hair. Moreover, longitudinal and radial diffusion phenomena may enlarge the spot where the incorporated substances are initially present [50].

By keeping most of the cited factors as known and controlled as possible, deductions about a missing parameter can be inferred: for example, the time at which a certain drug was taken, the compliance of a patient to follow a certain therapy, the period during which a worker was exposed to a certain industrial pollutant. Most of these queries are tentatively answered by means of segmental analysis, namely the reconstruction of the chronological sequence of the investigated substances' uptake from the analysis of small segments of a hair lock grown homogeneously. Due to the numbers of controlling factors that determine the hair content distribution, interpretation of segmental analysis should be conducted with caution, and much more experimental evidence needs to be collected in the future to improve its reliability further.

A key application of segmental hair analysis is represented by the forensic investigation of drug-facilitated crimes (DFC), in particular sex assaults [51]. An extensive review of the published literature in this field is presented in a chapter of this book. Taking into account only the major issues of these investigations, it is important to note that hair analysis is particularly problematic because (i) the incapacitating drug has allegedly been taken only once, leading to extremely low hair concentration and high analytical sensitivity requirements, (ii) the drug to be detected is potentially comprised within a list of tens of psychoactive substances and is generally not known in advance by the analyst [52–55], (iii) the circumstantial evidence is frequently disjointed, due to the amnesic effects of the drug. Thus, increasingly sensitive and multi-targeted analytical methods have recently been proposed to deal with these challenges [56–59]. While general recommendations for segmental analysis suggest the use of 10-30 mm segments [60,61] in order to cope with peak broadening phenomena (diffusion, differential hair growth, *etc.*) [50,62], more condensed segmentations are frequently useful in DFC investigations in order not to dilute the limited amount

of trapped drug into an excess of drug-free matrix [60],[63]. Indeed, under apparently comparable conditions, much lower concentrations were found in real forensic cases [57,64] than in single controlled administrations [43,44].

Another important application of segmental analysis with progressively increasing practice is related to therapeutic drug monitoring [65,66]. The pioneering work of Sato and co-workers showed that, for each patient, changes in the concentration of haloperidol and chlorpromazine concentration in hair segments corresponded to variations of the administered dose [67–69]. This observation disclosed the opportunity of monitoring dosage changes by following the drug concentration pattern in the sequence of hair segments, and to reveal episodes of noncompliance behavior. In rehabilitation programs, segmental analysis also reveals compliance to abstinence and the degree of non-compliance conduct. It is worth noting that a strong correspondence between drug dosage and hair concentration exists for the same patient, even for the substances that exhibited virtually no correlation at inter-individual level, due to genetic, personal, and behavioral factors (including hair melanin content) that modify on a large scale the absolute degree of hair incorporation.

During later research, therapeutic drug monitoring was applied to carbamazepine [70–72], selegiline [42], clozapine [73], buprenorphine [74–76] and methadone [77,78]. Buprenorphine received considerable interest recently because it is increasingly used to replace methadone in opiate addiction treatments. In its Suboxone® formulation, buprenorphine is mixed with naloxone, a strong opioid antagonist, to discourage drug diversion and abuse. Therefore, self-administration of Suboxone® is recurrently committed to the patients with a reliable history of compliance. For these patients, frequent urine tests to check compliance appear a pointless burden, while bi-monthly hair analysis represents a viable and cheap alternative. In the stabilization phase of buprenorphine maintenance therapy, the drug dosage is adjusted for each patient and frequently varied, making hair analysis highly valuable as a useful method to monitor these dosage changes. In a recent study, the summed buprenorphine and norbuprenorphine (its main metabolite) hair concentrations appeared to be highly sensitive to dosage changes and strictly correlated with them, along an intra-individual time scale [75]. The case of buprenorphine illustrates the reasons why hair analysis is likely to earn the chance to substitute urine and blood testing in many long-term drug monitoring programs in the future: more valuable information will be gained at less expense, as long as the preliminary treatment steps for hair samples receive some degree of automation, to reduce production costs and increase sample throughput. These themes will undoubtedly receive growing attention in the forthcoming years.

As in the case of administered prescriptions, segmental hair analysis is also being increasingly utilized to control compliance to illegal drug abstinence or occasional intake of drugs in the mid-

term [78]. Recently published papers have focused their interest on heroin [79], cocaine [80], cannabis [81,82], amphetamines [83], and new designer-drugs [84], showing a diverse pattern of crucial evidence that can be acquired from segmental hair analysis. Twenty-eight cases of lethal heroin overdose were investigated with the aim of disclosing whether previous abstinence from opiates played a role in the fatalities, as a result of reduced tolerance to the drug [79]. Segmental hair analysis allowed investigators to distinguish continuous consumers from previous abstinent subjects, and ascertain the recurrent intake of additional drugs. The two groups of deceased heroin users were found to have homogeneous morphine concentrations in post-mortem blood, proving that abstinence may not represent a critical factor in the fatalities, whereas the concurrent intake of other drugs possibly represents a more significant cause for the deaths [79]. The timeline of cocaine disappearance from growing hair was investigated within the implementation of rehabilitation programs involving drug abstinence. It was found that both cocaine and benzoylecgonine could be detected in the hair of former cocaine abusers for several months after abstinence started, suggesting caution in the assessment of alleged abstinence violation [80]. The absolute and relative hair concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC) and 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THCCOOH) were studied in both self-reporting and controlled administration of cannabis products, revealing limited dependence on consumption frequency [81], scattered absolute concentration values, and generalized decrease of THCCOOH from proximal to distal segments [82]. More precise correlation between consumption pattern and head hair concentration was found for methamphetamine [83] and the first reports that connect segmental hair analysis to the consumption history of cathinone- and pyrrolidinophenone-type new designer drugs in real cases of chronic intoxication have already been published [84,85]. Quite obviously, many more studies on segmental hair analysis for cannabimimetics, cathinones, phenethylamines, and other designer drugs, are anticipated for the future, as long as these new classes of substances find substantial diffusion among the population of drug consumers.

#### ***12.2.4. Individual factors***

Besides hair color and melanin content, many other personal factors, listed in Table 2, are expected to contribute to the variability of hair composition. Up to now, these factors have almost always been considered using an holistic approach, knowingly or unconsciously. This is to say that when the experimental results could not be predicted nor rationalized, then the reason was generally attributed to several aspects of individual variability, whose particular contributions cannot be ignored and whose effects should be considered as a whole. In the preceding pages, several

instances have been discussed, where the expected correlation between the dose of a certain substance (or exposure level to it) and hair concentration was not found or was extremely weak.

For practical applications, it is generally sufficient to know how reliable the conclusions that can be drawn from the experimental data are, and not to overestimate the predictability of the dependent variable, when most of the contributing factors are unknown. However, some of these “individual factors” have been extensively studied in the past, because they had been suspected of imparting substantial bias to the data. This has been the case for natural hair color and cosmetic treatments, whose effect has occasionally been overestimated, as discussed previously.

Whenever a decision has to be made on the basis of a cut-off value, these issues acquire crucial relevance. Quantitative determinations with legal impact are in fact the focal topic of most studies aiming to highlight the role of individual variability on the hair concentration of the targeted substance, as is the case for EtG and FAEE. This explains why recent research efforts are trying to single out the specific contribution of some other individual factors (ideally, each individual factor) to the overall data variability, in the attempt to build a more “parametric” modelling of uncertainty quantification. This endeavor also represents a tremendous open challenge for future research.

Among individual factors, genetic polymorphisms and various forms of genetic expression constitute the most difficult elements to deal with, because the source of information is generally missing, except for circumstances when some metabolic dysfunctions are evident. On the other hand, the first studies about the dependence of hair content on drug-metabolizing enzyme phenotyping have only started [86,87]. At least within ethnic groups, individuals are assumed to have relatively similar metabolic pathways toward toxicology relevant substances, but this similarity may not hold for specific drugs. Genotyping and genotyping characterization might be increasingly used in the future to uncover a potential source of inter-individual variability in hair analysis.

It has been sometimes questioned if the gender and/or the age of the controlled subjects may influence their metabolic response to the targeted substances. For example, the possible effect of gender differences on EtG and FAEE hair concentration has been recently examined by several scientists [88–90]. Gareri and co-workers studied a population of 199 female and 73 male subjects, particularly to verify if the hair EtG and FAEE cut-off values commonly adopted for a masculine-prevalent population could be appropriate also for a feminine cohort, taking into account that the application of cosmetic products and treatments are suspected to decrease EtG hair concentration and increase FAEE [88]. They concluded that colored hair actually exhibited partly biased results for EtG, and that slightly lower average EtG values were anyhow determined for females with respect to males, whereas comparable FAEE results were observed for the two populations.

Consequently, just for female subjects, they suggested (i) decreasing the hair EtG cut-off value to 20 pg/mg and (ii) combining EtG and FAEE determinations [88]. An almost opposite suggestion was made by Crunelle and co-workers, who investigated a cohort of 36 alcohol-dependent patients: they found the same strong correlation between alcohol consumption and hair EtG for males and females, and no gender effect [89]. They concluded that identical an evaluation scale and cut-off values can be used for both genders. Absence of gender differences for hair EtG response to alcohol intake was also confirmed in a retrospective study on a population of over 20,000 subjects (18,920 males and 1,373 females), prevalently examined for hair EtG within the driving license re-granting procedure [90]. Average alcohol consumption is however slightly lower for women in both the addict [89] and DUI offender [90] populations, leading to a lower incidence of positive samples among women [90]. The same study also considered the influence of the age of the controlled subjects on the hair-EtG positivity rate: the statistically significant higher incidence observed for the oldest subjects was nevertheless attributed to behavioral and habit differences (older people are more reluctant to refrain from alcohol than young, even when they are of being controlled), rather than to metabolic changes that intervene with aging [90]. A comparative study of hair EtG values after controlled alcohol ingestion on a statistically significant cohort of subjects of different ages would be necessary to clarify the possible existence of metabolic changes.

Also a variety of physio-pathological factors may also be taken into account in evaluating the results of hair analysis, especially when a decision is taken on the quantitation of a target biomarker. For example, Høiseith and co-workers recently studied how a decreased kidney function may alter the physiological level of hair EtG concentration [91]. Although very limited cases of positive EtG concentrations (HEtG > 30 pg/mg) in certain social low-alcohol drinkers were reported in the past, this study provides clear evidence that patients with serious renal disease may have hair EtG values far above the cut-off, even if they observe a low-alcohol diet. This evidence is possibly attributed to delayed excretion of EtG, resulting in increased hair incorporation [91].

While the hair concentration of cortisol [92,93], testosterone, and various metals [94,95] have been recently compared for obese subjects with respect to a reference population, analogous investigation has not yet been conducted for abused substances. Dependence of hair concentration on body mass index (BMI) is not expected for hydrophilic substances, such as EtG [90] and amphetamines, but highly hydrophobic substances are likely to accumulate into the fat tissues of the body to be released with delay, ultimately resulting in a modified chronological profile of hair concentration. This is surely the case for THC, but possibly also for FAEE, cocaine and morphine. However, the BMI effect on hair analysis has still to be investigated.

A seasonal factor has recently been disclosed for hair EtG, since the average EtG values measured in hair grown in the warm season are sensibly lower than those measured in the hair grown in the cold season [90]. The hypothetical reasons for this difference are various, and range from a lower consumption of alcohol in the warm months, or a more abundant perspiration leading to EtG dilution, up to a more recurrent removal of sweat, due to frequent showers and sea-bathing occurring during summer. Quite evidently, these reasons include effective lower exposure to the substance (i.e., no bias) together with climatic and behavioral factors, possibly associated with a bias. Thus, the seasonal effect has to be investigated further.

Other conceivable sources of individual variability should be considered under specific circumstances. These include the already cited cosmetic and hygiene habits, but also the mode of substance consumption (i.e., smoking, inhalation, injection, etc.) and its frequency (for example, regular drinking vs. binge drinking), together with the concomitant intake of physiological or metabolic interfering drugs or special diet regimes. Lastly, the clothes habitually worn might induce anomalous hair perspiration, especially hats, foulards, and scarves, and become a source of self-contamination, because the entire hair length is maintained in close contact with the head skin and its sebaceous and sweat emissions. The same might occur with pillows and armchairs with headrests, especially on the posterior vertex region of the head, which is commonly sampled [60].

Increasing interest is also devoted to the problem of collecting representative hair samples. For head hair, this problem has been quite extensively examined in the past, leading to the recommendation to collect hair from the posterior vertex region of the head, where the least variations of hair growth are observed [60]. However, recent concerns have been raised as to whether a single lock of hair is representative of the real hair concentration of the targeted substances [96], and how much variation in growth rate and sampling procedures might influence their distribution along the measured hair length [97]. Indeed, Dussy and co-workers recorded coefficients of variation up to 28% and 62%, respectively for EtG and caffeine, from ten hair locks collected from various sites of the skull of the same individuals [96]. Thus, it is always advisable to collect more than one hair lock and, in critical situations (for example, concentration close to the cut-off value), to repeat the analysis on further hair locks. LeBeau and co-workers evaluated the variability associated with growth rate of human head hair, as well as the ability to uniformly collect hair next to the scalp, the latter being affected by several sources of errors [97]. From both contributions, they deduced that a quite large range of uncertainty for chronological attribution exists, and that the first segment close to the cut actually corresponds to hair formed  $1.3 \pm 0.2$  to  $2.2 \pm 0.4$  months earlier [97].



Whenever it is impossible to collect a hair lock from the head, either because of complete baldness or because head hair has been subjected to strong cosmetic treatments, alternative sources of hair sampling should be found, possibly providing experimental results as close as possible to those that would have been obtained had head hair been available. However, non-homogeneous hair growth and impossibility to align hair shafts prevent any chronological assignment from non-head hair. For most drugs of abuse, accurate quantitation from non-head hair is generally not requested nor achievable and the analysis merely assesses the presence or absence of the drug, even if roughly comparable results were recently reported for amphetamines having been determined from head, pubic, and axillary hair collected from the same subjects [98]. More critical is the case of hair EtG, whose quantitative result has to be compared with cut-off values for excessive drinking [99]. Several recent studies [90,100–102] confirmed that pubic and axillary hair cannot substitute head hair for EtG determination, because they respectively over- and under-estimate the correct value, whereas chest hair [90,100,101], leg and arm hair [101], may adequately represent the correct head hair EtG content. Together with these matrices, beards can also be used to verify teetotalism [102]. It has also been recently assessed that head locks of hair which are longer than the prescribed 3-cm can be profitably utilized to measure EtG, because no significant washing-out effect occurs on more distal segments from regular hygiene practices [103]. Further research to investigate the chemical and physical properties of the keratin matrix, together with its three-dimensional architecture [104] is foreseen.

### **12.3. Innovative technologies and instrumental advancements**

Crucial issues of hair analysis in the past years were represented by the number of different analytical procedures needed to accomplish an exhaustive toxicological screening, and the minimal amount of hair needed to execute these procedures on separate aliquots, with sufficient sensitivity. The continuous innovation and rapid improvement of analytical instrumentation has radically modified this scenario in recent years, allowing increasingly comprehensive procedures to be run on progressively smaller hair aliquots. In particular, the unceasing development of new chromatographic and mass spectrometric technologies has considerably improved the separation of complex mixture components, their mass spectra resolution, and overall instrumental sensitivity, insomuch as to make the limit of quantification for most target substances substantially lower than their effective concentration in hair, at least for the majority of real applications [105,106]. Among innovations, the emergence of ultra-high pressure liquid chromatography, together with time-of-flight and Orbitrap mass analyzers, and a wide range of new devices for efficient ion generation,

accumulation, and transmission, epitomize the technological milestones toward the progressive accomplishments of hair analysis. On the other hand, the resulting high sensitivity has made even more mandatory the use of extreme care in both sample handling, to avoid contamination, and data interpretation, to avoid false positive judgments, as discussed in the preceding chapters.

### ***12.3.1. Broad-spectrum toxicological analysis***

Among the changes introduced by the new instrumental technologies, the issue of major impact, in terms of broad applicability, is possibly represented by the achievement of general toxicological analysis, with the aim of making it totally untargeted. Significant steps toward the completion of large multi-analyte and multi-class screenings by single analysis have already been taken by combining the high chromatographic resolution of UHPLC with the fast electronics and high sensitivity of modern triple quadrupole mass spectrometers. While in the years 2007-2009, several methods capable of screening 15-20 substances within a single LC run were proposed, already in 2012 the concomitant detection and quantification of an extended panel of 35 licit and illicit drugs and metabolites was developed on 50 mg of hair, achieving LOQ concentrations in the range of 0.5-100 pg/mg, and was applied to seventeen real forensic cases [56]. More recently, another LC-MS/MS method was developed to determine as many as 87 psychoactive drugs and metabolites in 20 mg of hair, reaching 0.3-45 pg/mg LOQ values, which was applied to real post-mortem specimen [107]. Similarly, a UHPLC-MS/MS protocol was used to determine 96 psychoactive drugs on a 10 mg hair specimen, yielding 2-50 pg/mg LOQ values for most analytes of interest [108]. Taking into account that for the latter methods LOD concentrations were found in the low pg/mg range for most psychoactive drugs, or even below 1 pg/mg for some specific substances, application of these methods in several forensic inquiries, including drug-facilitated crimes, appears to be at hand.

Fit-for-purpose multi-class and comprehensive UHPLC-MS/MS methods, with a relatively restricted panel of analytes, were developed to substitute multiple GC-MS procedures, with the final scope to improve sample throughput and decrease costs. These are crucial issues of increasing relevance in hair analysis, particularly for the application in workplace drug testing. In such a context, the main difficulty is to create a single analytical method adequate to determine simultaneously polar and basic (i.e. amphetamines), acidic (i.e. benzoylecgonine), and nonpolar (i.e. THC) drugs. Two recent papers undertook this task with similar outcomes: respectively 13 [109] and 16 [110] drugs of abuse and metabolites, including THC, were separated in about 5 min, and detected after MS/MS analysis down to LOQ concentrations of 20-80 pg/mg, using triple

quadrupole instruments. A further published method for hair analysis determined as many as 33 drugs of abuse and metabolites (without THC) in less than 9 min, with LOD values ranging from 6 to 63 pg/mg [111].

The increasing performances of high-resolution mass spectrometers, in terms of sensitivity and acquisition speed, have made these instruments potentially capable of executing a truly general toxicological analysis on hair samples, allowing factual untargeted screening and retrospective reexamination of acquired data, following the appearance of upcoming investigation elements. As a matter of fact, in most drug-facilitated crimes, acute intoxications, and post-mortem investigations, the intoxicating substance cannot be anticipated, or even the actual occurrence of an intoxicating agent is doubtful. Thus, negative results from a targeted screening leave the chance open that an untargeted substance is present in the investigated sample.

Untargeted screening of hair samples performed by hybrid QTOF MS/MS devices, or similar instrumental arrangements, requires some forms of data-dependent [112] or data-independent [111,112] acquisition software, and the availability of large tandem mass spectra libraries [110,113]. For example, MS and MS/MS modes of acquisition are alternated within short cycle periods, where the preliminary low-resolution MS spectrum is used to identify the most abundant precursor ions at a certain retention time and the subsequent high-resolution MS/MS spectra of the automatically-selected precursors (recorded upon collisional activation) are compared with dedicated libraries of collision induced dissociation spectra of toxicologically relevant substances. Other strategies of data collection and library inquiry have been implemented on single- and double-stage mass spectrometers and software of increasing sophistication will be made commercially available in the near future to accomplish these tasks. However, the benefits of comprehensive screening are still somewhat compromised by a decreased sensitivity compared to targeted screening protocols [115]. This represents a critical drawback when a limited amount of samples are available, as in hair analysis. For this reason, broad-spectrum targeted screening methods have also been developed for high-resolution mass spectrometers, with which the collection of accurate mass signals is exploited to provide unequivocal identification of the targeted drugs.

Progressively higher performances were obtained as long as improved instrumental technologies were made commercially available. For example, 52 licit and illicit drugs were targeted in 10 mg hair samples using a single TOF instrument, that allowed to reach LOD concentrations in the range 10-100 pg/mg and LOQs around 50-200 pg/mg [116]. In the same period, other groups used Orbitrap mass spectrometers to complete toxicological screenings: in one study, 28 substances were monitored in 2.5 mg hair aliquots with LOQ values ranging from 100

pg/mg to 500 pg/mg [117]; in another study, an hybrid linear ion trap – Orbitrap tandem mass spectrometer was used to determine a restricted panel of stimulant drugs on extremely small aliquots (0.2 mg) of hair samples, also yielding LOQ values in the range 100-500 ng/mg [118]. More recently, LOQ concentrations in the tens of pg/mg range were obtained for a wide panel of new psychoactive substances, using 100 mg of hair and an analytical method based on an hybrid Q-TOF mass spectrometer [48]. The comparison of these figures-of-merit with those obtained from triple quadrupole instruments, previously reported, apparently indicates that high-resolution mass analyzers, even in the hybrid configuration, still provide slightly lower sensitivity than triple quadrupole instruments for targeted screening investigations on hair samples. However, the sensitivity gap between the two classes of instruments observed in the scientific literature is being progressively filled with the introduction of improved technologies for ion- and energy-focusing, ion accumulation, and synchronization of ion storage and mass detection processes, and reflects their delayed widespread dissemination into the toxicology laboratories.

### ***12.3.2. Highly-demanding investigations***

Besides highly-general screening, also highly-specific and/or demanding determinations characterize the incipient frontier of hair analysis. The investigation following drug-facilitated crimes represents a good example of a highly-demanding objective for hair analysis, as previously mentioned. Further examples of highly-demanding investigations are (i) the detection of certain drug metabolites at particularly low concentration, necessary to exclude external contamination; (ii) the quantitative discrimination of enantiomers, when their pharmacological activity is different or only one stereoisomer produces psychoactive effects; (iii) the determination of licit and illicit drugs on extremely small hair aliquots, or even on a single hair.

Among the drug metabolites whose determination in hair samples is particularly problematic, the most extensively studied is certainly nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH), whose hair concentration is frequently below 1 pg/mg, and cut-off values to prove cannabis abuse are set at 50-100 fg/mg. While the traditional methods to determine THC-COOH in hair are based on gas chromatography - electron capture negative ionization – tandem mass spectrometry (GC-ECNI-MS/MS), after chemical derivatization, new alternative approaches have been recently proposed to achieve extremely low detection limits. These include GC $\times$ GC-MS [119], surface-activated chemical ionization combined with electrospray ionization and LC-MS [120], negative-ion electrospray ionization LC-MS/MS of the unmodified THC-COOH [121] or its methyl ester, obtained after selective derivatization that leaves the hydroxyl group unchanged and

still available for negative ionization [122]. The latter approach is particularly skillful, because it entirely removes the interference of fatty acids, and opens the way to further reduction of the THC-COOH detection limit, as long as the chromatographic and mass spectrometric instrumentation keeps improving. The discrimination between drug intake and external contamination by means of metabolites detection also applies to cocaine, but the sensitivity requirements for the determination of cocaine metabolites are less severe, and consequently the struggle to set up innovative analytical methods is comparatively less forceful [123].

Chiral drug analysis is another topic of increasing investigation because of its relevance in clinical and forensic toxicology. Up to now, a large number of these studies has been conducted on blood and urine specimen [124], but very few on the keratin matrix, despite its unique property of providing a time-integrated perspective of drug intake. Thus, more frequent recourse to chiral separation in hair analysis is expected in the forthcoming years. Stereoisomer discrimination has been completed for amphetamines present in hair samples by both GC-MS [123,124] and LC-MS [127] methods. GC-MS analysis of amphetamines enantiomers was conducted after derivatization with (S)-heptafluorobutyrylpropyl chloride, which generated couples of diastereoisomers, that could be separated with a non-chiral stationary phase and ionized in the electron-capture negative ion mode [125]. Similarly, a new chiral derivatization agent of improved efficiency was synthesized (namely, (2S,4R)-N-heptafluorobutyryl-4-heptafluorobutoxyloxy-propyl chloride) and utilized under comparable conditions [126]. For the five amphetamines most present on the illegal market, LOQ values in the range 7-150 pg/mg were obtained with the former derivatization agent, but LODs were reduced to 2-9 pg/mg when the new and optimized derivatization agent was applied to the hair extracts [126]. Derivatization of the amine group of amphetamines was applied also before LC separation to produce a 5-fold sensitivity increase, but a non-chiral reagent was used (i.e., trifluoroacetic anhydride); thus, the resulting derivative enantiomers had still to be separated on a chiral stationary phase [127].

The enantioselective metabolism of levomethorphan (a narcotic drug) and its discrimination from the enantiomeric dextromethorphan (an antitussive medicine), was investigated on various biological samples including hair, collected from rats [128]. The LC-MS/MS analytical procedure involved the use of a chiral LC column and achieved complete separation of enantiomers for the parent substances and their O-demethyl and N, O-didemethyl metabolites in 12 minutes, showing that the drug metabolism does not induce its racemization. This represents a crucial aspect for the prospective application to human hair, in view of toxicological applications [128]. Also the enantioselective metabolism of methadone, commonly administered as a racemic mixture, was demonstrated in a study founded on the setup of a dedicated LC-MS/MS method, where three

couples of enantiomers (methadone and its two main metabolites) were extracted from hair samples and chromatographically separated on a chiral column [129]. This complex task was proficiently accomplished by using factorial analysis experimental design and artificial neural networks to optimize the chromatographic separation from response surfaces analysis [129]. As a matter of fact, the widespread use of multivariate chemometric tools represents another challenging perspective for hair analysis, both to deduce optimal experimental conditions and planning from a reduced set of preliminary experiments, as in the study previously cited, and to interpret the resultant data on the basis of sound statistical principles. For example, principal component analysis can be extensively employed for data mining [130], while statistical discriminant analysis can be exploited to make predictions and estimate likelihood ratios to distinguish real drug consumption from external contamination of hair [131]. The Bayesian concept of likelihood ratio was also proposed to support the interpretation of hair testing for alcohol abuse with solid probabilistic foundation [132].

### ***12.3.3. Minute hair availability and single hair analysis***

Another demanding request for future hair analysis is the opportunity to determine drugs on extremely minute amounts of hair sample, down to the limit of a single hair. Currently, single-hair analysis is no longer a simple wishful dream, but is nowadays a concrete objective, persistently pursued with increasing success. However, the need to operate on tiny amounts of keratin matrix is frequently combined with a requisite of low detection limits, in terms of concentration, making single hair analysis not yet adequate to fulfill both requirements. The most promising approach to decrease the amount of sampled hair and the drug detection limits at the same time, utilized a microfluidic chip-based nano-HPLC system, coupled to tandem mass spectrometry [130,131]. In the most recent publication, the nano-LC-MS/MS technique was applied to the determination of 14 illicit drugs and metabolites extracted from only 2 mg of hair, and obtained low detection limits for all analytes, that ranged from 0.10 pg/mg to 0.75 pg/mg [134]. A similar procedure, involving a chromatographic run time of 15 min with an eluent flow-rate of 4  $\mu$ L/min, was applied to the determination of ketamine and norketamine in 10 mg of hair. Again, LODs of 0.5 and 1.0 pg/mg were obtained, with very little solvent consumption [133].

Single hair analysis was recently conducted to detect both organic and inorganic analytes, mostly for toxicological purposes. The content of heavy metals (Cd and Pb) in a single hair was investigated by coupling of a tungsten coil electro-thermal vaporizer with an argon-hydrogen flame for atomic fluorescence spectrometry. In particular, an outstanding absolute detection limit of 50 fg was observed for Cd [135]. In another study, a wide panel of essential and toxic metals were

determined in single hair by laser ablation – inductively coupled plasma – mass spectrometry, reaching unequal LODs in the range 1-900 pg/mg [136]. The advantage of laser ablation lies in its micrometric spatial resolution, that allows investigation of the metal longitudinal distribution along the hair length.

The detection of drugs on single hairs has been handled both by (i) direct spatially-resolved exposure to impinging particles such as laser photons or electrospray droplets and (ii) preliminary minute segmentation of the hair followed by application of dedicated small-volume extraction and treatment methods for each hair segment. In both approaches, the absolute amount of drug to be detected is extremely small and, consequently, sensitivity limitations exist, that fundamentally restrict single hair investigations to targeted analysis. On the other hand, the analysis of a single hair furnishes a more detailed chronological profile of drug intake than a hair lock, because several confounding factors are not present, such as alternating hair growth phases and incorrect alignment of hair bundles during sampling. The absence of these smoothing effects on peak concentrations partly compensate for the reduced sensitivity due to minimal sampling.

Thieme and Sachs introduced single hair segmental analysis in 2007, to investigate a case of repeated clozapine poisoning [137]. Fine segmentation (1.0-2.5 mm) of individual hairs provided an extremely detailed chronology of clozapine administration, with a resolution within a few days and a detection limit of about 1 pg/mg. This study also revealed that synchronization of sharp clozapine blood and hair peaks occurred, thus showing the marginal role of drug incorporation from sweat and sebum and excluding the incidence of any longitudinal diffusion of the drug along the hair shaft. Circumstantial evidence also allowed calculation of the hair growth rate for the investigated subject [137]. The same scientists recently expanded the application of single hair segmental analysis to the screening of further psychoactive substances, potentially used to perpetrate drug-facilitated crimes [58]. The proposed investigation strategy for DFC cases involved the preliminary screening of 183 substances on a hair lock of adequate weight, followed by fine segmental analysis on single hairs, targeted to the drugs whose presence was ascertained. Segments of 0.25-0.50 mm, and weight of 10-50 µg, proved optimal to balance sensitivity, quantitative accuracy, and time resolution of this chronological profiling. Quite obviously, all the analytical steps had to be optimized to work on a microscopic scale [58].

Since 2011, matrix-assisted laser desorption/ionization (MALDI) has been proposed to obtain a punctual analysis of single hair, resulting in a mass-spectrometric image of drug distribution along the hair shaft [135–137]. Even before, MALDI was applied to pulverized hair (1.0-2.5 mg) to execute rapid and high-throughput screening of hair samples for cocaine and metabolites [138,139]. The first method developed to prepare single hairs for MALDI-MS imaging involved attaching the

hair to a glass slide using a conductive carbon adhesive tape, then manually cutting the hair shaft lengthwise using a razor and a microscope and applying  $\alpha$ -cyano-4-hydroxycinnamic acid as the MALDI matrix. After completing this procedure, the arranged hair was exposed to the laser beam, and the desorbed ions were detected either with a time-of-flight or a Fourier transform ion cyclotron resonance mass analyzer. The method was applied to create highly resolved drug concentration profiles of the hair taken from amphetamine abusers [135,136]. The complex manual step of microscopic hair slicing was avoided in the MS imaging study conducted on the hair collected from cocaine abusers, considerably shortening the sample preparation time [140]. Cocaine and its main metabolites were nevertheless determined with a limit of detection of about 5 ng/mg (corresponding to 20 fg of cocaine per laser shot), adequate for chronological drug profiling of the hair of habitual drug consumers, not for the detection of a single and episodic intake. Both axial and radial diffusion of the analytes during the acquisition time proved to be modest, allowing a temporal resolution of hair growth of a few days [140]. In a subsequent study, the use of a MALDI-MS/MS instrument with high resolution capability in the second stage of mass analysis allowed the registration of the chronological profile for both the parent drug and two metabolites in the hair of an episodic cocaine consumer [143]. However, it was observed that, while two hairs furnished similar profiles, two others from the same subject turned out totally negative to the presence of cocaine. This observation alerted researchers to the fact that the results from single hair analysis have to be carefully evaluated in the light of different growth phases, which might produce false negative results if the intake occurs during the quiescent telogen and catagen periods [143],[144]. Image profile was determined also for ketamine in three hairs out of four collected from a single individual. A MALDI source interfaced to 9.4 Tesla FTICR mass spectrometer was used to this purpose. Gentle scraping using a scalpel somewhat damaged the hair surface, so as to allow the incorporated substances to be more easily desorbed outside the keratin structure [144].

Unlike clozapine [137], single hair analysis of samples collected 30 days after unique intake of tilidine revealed quite homogeneous distribution of the drug along the hair length and large concentration differences among five hairs collected from the same subject. These two observations suggested that these hairs had been randomly coated on their external surface by the sweat containing tilidine shortly after its administration; then, the tilidine coated at the surface was incorporated inside the keratin structure, simulating chronic administration [145]. The dissimilar hair distribution obtained from single intake of clozapine and tilidine (sharp concentration peak vs. homogeneous distribution) is likely to reflect the different predominant way by which these drugs are incorporated, respectively blood or sweat. Consequently, the practical utility of highly-resolved segmental hair analysis becomes questionable when the prevalent way leading to drug incorporation



is sweat, suggesting the need (i) to use extreme care in the interpretation of the inherent data and (ii) to conduct further research on the mechanisms of drug transfer from biological fluids to hair, for each class of substances.

Further direct ionization methods prior to MS analysis, including low temperature plasma ionization (LTP) [146], desorption electrospray ionization (DESI) [147], and direct analysis in real time (DART) [148] had been proposed to perform fast and cheap drug screening on hair locks and hair extracts. However, the first attempts to work directly on the keratin material failed, and some form of extraction proved to be necessary before applying ambient ionization to the extracts [143,144]. The more recent study showed the successful application of DART to the detection of THC directly on hair locks collected from chronic drug abusers [148]. Although the instrumentation used was not ideal to achieve the highest sensitivity toward a single target compound, this study demonstrated the potential of the DART-MS technique for the fast screening of hair samples. Easily achievable improvements are predictable using dedicated mass spectrometers and further refinement of the experimental conditions.

These are only few of the technological advancements expected in the forthcoming years that will influence the advancements of hair analysis. A weak point of almost any current analytical method is the extensive need of manual operations during the initial hair sample preparation. This weakness limits the widespread application of hair analysis and has a major impact on the final cost of any analytical protocol. Therefore, decisive technological improvements are waited in the automation of hair sample processing, in order to reduce the manual intervention and its associated cost and variability, to assure more constant operating conditions, and to increase sample throughput. Under such conditions, the role of hair analysis in the assessment of human exposure to a variety of drugs and/or toxic substances will rapidly grow in any field of clinical and toxicological domains, making hair a customary biological matrix to investigate, as is presently true for urine and blood.

## REFERENCES

1. Wennig, R. (2000). Potential problems with the interpretation of hair analysis results. *Forensic Sci. Int.* **107**, 5–12.
2. Kidwell, D. A., Lee, E. H. and DeLauder, S. F. (2000). Evidence for bias in hair testing and procedures to correct bias. *Forensic Sci. Int.* **107**, 39–61.
3. Musshoff, F. and Madea, B. (2007). Analytical pitfalls in hair testing. *Anal. Bioanal. Chem.* **388**, 1475–94.

4. Musshoff, F. and Madea, B. (2007). New trends in hair analysis and scientific demands on validation and technical notes. *Forensic Sci. Int.* **165**, 204–15.
5. Tagliaro, F., Bortolotti, F., Viel, G. and Ferrara, S. D. (2011). Caveats against an improper use of hair testing to support the diagnosis of chronic excessive alcohol consumption, following the “Consensus” of the Society of Hair Testing 2009 [Forensic Science International 196 (2010) 2]. *Forensic Sci. Int.* **207**, 69–71.
6. LeBeau, M. A. and Montgomery, M. A. (2009). Considerations on the utility of hair analysis for cocaine. *J. Anal. Toxicol.* **33**, 343–4.
7. Pragst, F., Sachs, H. and Kintz, P. (2010). Hair analysis for cocaine continues to be a valuable tool in forensic and clinical toxicology. *J. Anal. Toxicol.* **34**, 354–6.
8. Ettlinger, J., Kirchen, L. and Yegles, M. (2014). Influence of thermal hair straightening on ethyl glucuronide content in hair. *Drug Test. Anal.* **6 Suppl 1**, 74–7.
9. Musshoff, F., Brockmann, C., Madea, B., Rosendahl, W. and Piombino-Mascalì, D. (2013). Ethyl glucuronide findings in hair samples from the mummies of the Capuchin Catacombs of Palermo. *Forensic Sci. Int.* **232**, 213–7.
10. Skopp, G., Pötsch, L. and Mauden, M. (2000). Stability of cannabinoids in hair samples exposed to sunlight. *Clin. Chem.* **46**, 1846–8.
11. Favretto, D., Tucci, M., Monaldi, A., Ferrara, S. D. and Miolo, G. (2014). A study on photodegradation of methadone, EDDP, and other drugs of abuse in hair exposed to controlled UVB radiation. *Drug Test. Anal.* **6 Suppl 1**, 78–84.
12. Gerace, Enrico; Veronesi, Agnese; Martra, Gianmario; Salomone, Alberto; Vincenti, M. Study of the incorporation of cocaine in damaged hair samples. *submitted*.
13. Hill, V., Loni, E., Cairns, T., Sommer, J. and Schaffer, M. (2014). Identification and analysis of damaged or porous hair. *Drug Test. Anal.* **6 Suppl 1**, 42–54.
14. Cirimele, V., Kintz, P. and Mangin, P. (1995). Drug concentrations in human hair after bleaching. *J. Anal. Toxicol.* **19**, 331–2.
15. Jurado, C., Kintz, P., Menéndez, M. and Repetto, M. (1997). Influence of the cosmetic treatment of hair on drug testing. *Int. J. Legal Med.* **110**, 159–63.
16. Skopp, G., Pötsch, L. and Moeller, M. R. (1997). On cosmetically treated hair--aspects and pitfalls of interpretation. *Forensic Sci. Int.* **84**, 43–52.
17. Agius, R. (2014). Utility of coloured hair for the detection of drugs and alcohol. *Drug Test. Anal.* **6 Suppl 1**, 110–9.
18. Morini, L., Zucchella, A., Polettini, A., Politi, L. and Groppi, A. (2010). Effect of bleaching on ethyl glucuronide in hair: an in vitro experiment. *Forensic Sci. Int.* **198**, 23–7.

19. Martins, L. F., Yegles, M., Thieme, D. and Wennig, R. (2008). Influence of bleaching on the enantiomeric disposition of amphetamine-type stimulants in hair. *Forensic Sci. Int.* **176**, 38–41.
20. Baeck, S., Han, E., Chung, H. and Pyo, M. (2011). Effects of repeated hair washing and a single hair dyeing on concentrations of methamphetamine and amphetamine in human hairs. *Forensic Sci. Int.* **206**, 77–80.
21. Cuypers, E., Flinders, B., Bosman, I. J., Lusthof, K. J., Van Asten, A. C., Tytgat, J. and Heeren, R. M. a. (2014). Hydrogen peroxide reactions on cocaine in hair using imaging mass spectrometry. *Forensic Sci. Int.* **242C**, 103–110.
22. Hill, V., Cairns, T. and Schaffer, M. (2008). Hair analysis for cocaine: factors in laboratory contamination studies and their relevance to proficiency sample preparation and hair testing practices. *Forensic Sci. Int.* **176**, 23–33.
23. Stout, P. R., Roper-Miller, J. D., Baylor, M. R. and Mitchell, J. M. (2007). Morphological changes in human head hair subjected to various drug testing decontamination strategies. *Forensic Sci. Int.* **172**, 164–70.
24. Binz, T. M., Baumgartner, M. R. and Kraemer, T. (2014). The influence of cleansing shampoos on ethyl glucuronide concentration in hair analyzed with an optimized and validated LC-MS/MS method. *Forensic Sci. Int.* doi: 10.1016/j.forsciint.2014.07.021.
25. Röhrich, J., Zörntlein, S., Pötsch, L., Skopp, G. and Becker, J. (2000). Effect of the shampoo Ultra Clean on drug concentrations in human hair. *Int. J. Legal Med.* **113**, 102–6.
26. Kidwell, D. A., Lee, E. H. and DeLauder, S. F. (2000). Evidence for bias in hair testing and procedures to correct bias. *Forensic Sci. Int.* **107**, 39–61.
27. Borges, C. R., Roberts, J. C., Wilkins, D. G. and Rollins, D. E. (2003). Cocaine, benzoylecgonine, amphetamine, and N-acetylamphetamine binding to melanin subtypes. *J. Anal. Toxicol.* **27**, 125–34.
28. Claffey, D. J. and Ruth, J. a. (2001). Amphetamine adducts of melanin intermediates demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Chem. Res. Toxicol.* **14**, 1339–44.
29. Kharbouche, H., Steiner, N., Morelato, M., Staub, C., Boutrel, B., Mangin, P., Sporkert, F. and Augsburg, M. (2010). Influence of ethanol dose and pigmentation on the incorporation of ethyl glucuronide into rat hair. *Alcohol* **44**, 507–14.
30. Kulaga, V., Velazquez-Armenta, Y., Aleksa, K., Vergee, Z. and Koren, G. (2009). The effect of hair pigment on the incorporation of fatty acid ethyl esters (FAEE). *Alcohol Alcohol.* **44**, 287–92.
31. Lee, S., Han, E., Kim, E., Choi, H., Chung, H., Oh, S. M., Yun, Y. M., Jwa, S. H. and Chung, K. H. (2010). Simultaneous quantification of opiates and effect of pigmentation on its deposition in hair. *Arch. Pharm. Res.* **33**, 1805–11.

32. Kim, J., In, S., Park, Y., Park, M., Kim, E. and Lee, S. (2013). Deposition of JWH-018, JWH-073 and their metabolites in hair and effect of hair pigmentation. *Anal. Bioanal. Chem.* **405**, 9769–78.
33. Kelly, R. C., Mieczkowski, T., Sweeney, S. a and Bourland, J. a. (2000). Hair analysis for drugs of abuse. Hair color and race differentials or systematic differences in drug preferences? *Forensic Sci. Int.* **107**, 63–86.
34. Mieczkowski, T. and Newel, R. (2000). Statistical examination of hair color as a potential biasing factor in hair analysis. *Forensic Sci. Int.* **107**, 13–38.
35. Nordlund, James J; Boissy, Raymond E; Hearing, Vincent J; King, Richard A; Oetting, William S; Ortonne, J.-P. (2006). *The Pigmentary System : Physiology and Pathophysiology* 2nd ed., p. 1328.
36. Rollins, D. E., Wilkins, D. G., Krueger, G. G., Augsburg, M. P., Mizuno, A., O'Neal, C., Borges, C. R. and Slawson, M. H. (2003). The effect of hair color on the incorporation of codeine into human hair. *J. Anal. Toxicol.* **27**, 545–51.
37. Kronstrand, R., Förstberg-Peterson, S., Kågedal, B., Ahlner, J. and Larson, G. (1999). Codeine concentration in hair after oral administration is dependent on melanin content. *Clin. Chem.* **45**, 1485–94.
38. Scheidweiler, K. B., Cone, E. J., Moolchan, E. T. and Huestis, M. A. (2005). Dose-related distribution of codeine, cocaine, and metabolites into human hair following controlled oral codeine and subcutaneous cocaine administration. *J. Pharmacol. Exp. Ther.* **313**, 909–15.
39. Mieczkowski, T. and Kruger, M. (2007). Interpreting the color effect of melanin on cocaine and benzoylecgonine assays for hair analysis: brown and black samples compared. *J. Forensic Leg. Med.* **14**, 7–15.
40. Kronstrand, R., Roman, M., Hedman, M., Ahlner, J. and Dizdar, N. (2007). Dose–hair concentration relationship and pigmentation effects in patients on low-dose clozapine. *Forensic Sci. Med. Pathol.* **3**, 107–114.
41. Rothe, M., Pragst, F., Thor, S. and Hunger, J. (1997). Effect of pigmentation on the drug deposition in hair of grey-haired subjects. *Forensic Sci. Int.* **84**, 53–60.
42. Kronstrand, R; Ahlner, J; Dizdar, N; Larson, G. (2003). Quantitative analysis of desmethylselegiline, methamphetamine and amphetamine in hair and plasma from Parkinson patients on long-term selegiline medication. *J. Anal. Toxicol.* **27**, 135–141.
43. Cui, X., Xiang, P., Zhang, J., Shi, Y., Shen, B. and Shen, M. (2013). Segmental hair analysis after a single dose of zolpidem: comparison with a previous study. *J. Anal. Toxicol.* **37**, 369–75.
44. Villain, M., Chèze, M., Tracqui, A., Ludes, B. and Kintz, P. (2004). Windows of detection of zolpidem in urine and hair: application to two drug facilitated sexual assaults. *Forensic Sci. Int.* **143**, 157–61.

45. Appenzeller, B. M. R., Schuman, M., Yegles, M. and Wennig, R. (2007). Ethyl glucuronide concentration in hair is not influenced by pigmentation. *Alcohol Alcohol.* **42**, 326–7.
46. Hutter, M., Kneisel, S., Auwärter, V. and Neukamm, M. a. (2012). Determination of 22 synthetic cannabinoids in human hair by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **903**, 95–101.
47. Salomone, a, Gerace, E., D’Urso, F., Di Corcia, D. and Vincenti, M. (2012). Simultaneous analysis of several synthetic cannabinoids, THC, CBD and CBN, in hair by ultra-high performance liquid chromatography tandem mass spectrometry. Method validation and application to real samples. *J. Mass Spectrom.* **47**, 604–10.
48. Gottardo, R., Sorio, D., Musile, G., Trapani, E., Seri, C., Serpelloni, G. and Tagliaro, F. (2014). Screening for synthetic cannabinoids in hair by using LC-QTOF MS: a new and powerful approach to study the penetration of these new psychoactive substances in the population. *Med. Sci. Law* **54**, 22–7.
49. Salomone, a, Luciano, C., Di Corcia, D., Gerace, E. and Vincenti, M. (2014). Hair analysis as a tool to evaluate the prevalence of synthetic cannabinoids in different populations of drug consumers. *Drug Test. Anal.* **6**, 126–34.
50. Kintz, P. (2013). Issues about axial diffusion during segmental hair analysis. *Ther. Drug Monit.* **35**, 408–10.
51. Scott, K. S. (2009). The use of hair as a toxicological tool in DFC casework. *Sci. Justice* **49**, 250–3.
52. Scott-Ham, M. and Burton, F. C. (2005). Toxicological findings in cases of alleged drug-facilitated sexual assault in the United Kingdom over a 3-year period. *J. Clin. Forensic Med.* **12**, 175–86.
53. Hall, J. A. and Moore, C. B. T. (2008). Drug facilitated sexual assault--a review. *J. Forensic Leg. Med.* **15**, 291–7.
54. Madea, B. and Musshoff, F. (2009). Knock-out drugs: their prevalence, modes of action, and means of detection. *Dtsch. Arztebl. Int.* **106**, 341–7.
55. Parkin, M. C. and Brailsford, A. D. (2009). Retrospective drug detection in cases of drug-facilitated sexual assault: challenges and perspectives for the forensic toxicologist. *Bioanal.* **1**, 1001–13.
56. Lendoiro, E., Quintela, O., de Castro, A., Cruz, A., López-Rivadulla, M. and Concheiro, M. (2012). Target screening and confirmation of 35 licit and illicit drugs and metabolites in hair by LC-MSMS. *Forensic Sci. Int.* **217**, 207–15.
57. Salomone, A., Gerace, E., Di Corcia, D., Martra, G., Petrarulo, M. and Vincenti, M. (2012). Hair analysis of drugs involved in drug-facilitated sexual assault and detection of zolpidem in a suspected case. *Int. J. Legal Med.* **126**, 451–9.

58. Thieme, D., Baumer, C., Sachs, H. and Teske, J. (2013). Screening and long-term retrospection for psychoactive drugs in presumptive drug-facilitated crimes using segmented single hairs. *Drug Test. Anal.* **5**, 736–40.
59. Maublanc, J., Dulaurent, S., Morichon, J., Lachâtre, G. and Gaulier, J.-M. (2014). Identification and quantification of 35 psychotropic drugs and metabolites in hair by LC-MS/MS: application in forensic toxicology. *Int. J. Legal Med.* doi: 10.1007/s00414-014-1005-1.
60. Cooper, G. a a, Kronstrand, R. and Kintz, P. (2012). Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci. Int.* **218**, 20–4.
61. Kintz, P. (2007). Bioanalytical procedures for detection of chemical agents in hair in the case of drug-facilitated crimes. *Anal. Bioanal. Chem.* **388**, 1467–74.
62. Xiang, P., Sun, Q., Shen, B., Chen, P., Liu, W. and Shen, M. (2011). Segmental hair analysis using liquid chromatography-tandem mass spectrometry after a single dose of benzodiazepines. *Forensic Sci. Int.* **204**, 19–26.
63. Jakobsson, G. and Kronstrand, R. (2014). Segmental analysis of amphetamines in hair using a sensitive UHPLC-MS/MS method. *Drug Test. Anal.* **6 Suppl 1**, 22–9.
64. Kintz, P., Villain, M., Dumestre-Toulet, V. and Ludes, B. (2005). Drug-facilitated sexual assault and analytical toxicology: the role of LC-MS/MS A case involving zolpidem. *J. Clin. Forensic Med.* **12**, 36–41.
65. Beumer, J. H., Bosman, I. J. and Maes, R. A. (2001). Hair as a biological specimen for therapeutic drug monitoring. *Int. J. Clin. Pract.* **55**, 353–7.
66. García-Algar, Óscar; Pichini, Simona; de la Torre, R. (2006). Clinical Applications of Hair Analysis. In "Analytical and Practical Aspects of Drug Testing in Hair" (Kintz, P., ed.), pp. 201–22.
67. Uematsu, T., Sato, R., Suzuki, K., Yamaguchi, S. and Nakashima, M. (1989). Human scalp hair as evidence of individual dosage history of haloperidol: method and retrospective study. *Eur. J. Clin. Pharmacol.* **37**, 239–44.
68. Sato, R., Uematsu, T., Yamaguchi, S. and Nakashima, M. (1989). Human scalp hair as evidence of individual dosage history of haloperidol: prospective study. *Ther. Drug Monit.* **11**, 686–91.
69. Sato, H., Uematsu, T., Yamada, K. and Nakashima, M. (1993). Chlorpromazine in human scalp hair as an index of dosage history: comparison with simultaneously measured haloperidol. *Eur. J. Clin. Pharmacol.* **44**, 439–44.
70. Kintz, P., Marescaux, C. and Mangin, P. (1995). Testing human hair for carbamazepine in epileptic patients: Is hair investigation suitable for drug monitoring? *Hum. Exp. Toxicol.* **14**, 812–815.
71. Williams, J., Patsalos, P. N. and Wilson, J. F. (1997). Hair analysis as a potential index of therapeutic compliance in the treatment of epilepsy. *Forensic Sci. Int.* **84**, 113–122.

72. Williams, J. (1999). The assessment of therapeutic compliance based upon the analysis of drug concentration in hair. In "Drug testing technology" (Mieczkowski, T., ed.), pp. 1–32.
73. Shen, M., Xiang, P., Wu, H., Shen, B. and Huang, Z. (2002). Detection of antidepressant and antipsychotic drugs in human hair. *Forensic Sci. Int.* **126**, 153–161.
74. Skopp, G., Kniest, A., Haisser, J., Mann, K. and Hermann, D. (2011). Buprenorphine and norbuprenorphine findings in hair during constant maintenance dosage. *Int. J. Legal Med.* **125**, 277–81.
75. Pirro, V., Fusari, I., Di Corcia, D., Gerace, E., De Vivo, E., Salomone, A. and Vincenti, M. (2014). Hair analysis for long-term monitoring of buprenorphine intake in opiates withdrawal. *Ther. Drug Monit.* doi: 10.1097/FTD.0000000000000078.
76. Belivanis, S., Tzatzarakis, M. N., Vakonaki, E., Kovatsi, L., Mantsi, M., Alegakis, A., Kavvalakis, M. P., Vynias, D. and Tsatsakis, A. M. (2014). Buprenorphine and norbuprenorphine levels in head hair samples from former heroin users under Suboxone® treatment. *Drug Test. Anal.* **6 Suppl 1**, 93–100.
77. Kelly, T., Doble, P. and Dawson, M. (2005). Chiral analysis of methadone and its major metabolites (EDDP and EMDP) by liquid chromatography-mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **814**, 315–23.
78. Seldèn, T., Berglund, L., Druid, H., Håkansson, A. and Kronstrand, R. (2014). O9: Segmental hair analysis from patients in opiate substitution treatment is useful to investigate drug use history, abstinence and compliance with treatment. *Toxicol. Anal. Clin.* **26**, S8–S9.
79. Druid, H., Strandberg, J. J., Alkass, K., Nyström, I., Kugelberg, F. C. and Kronstrand, R. (2007). Evaluation of the role of abstinence in heroin overdose deaths using segmental hair analysis. *Forensic Sci. Int.* **168**, 223–6.
80. Garcia-Bournissen, F., Moller, M., Nesterenko, M., Karaskov, T. and Koren, G. (2009). Pharmacokinetics of disappearance of cocaine from hair after discontinuation of drug use. *Forensic Sci. Int.* **189**, 24–7.
81. Huestis, M. A., Gustafson, R. A., Moolchan, E. T., Barnes, A., Bourland, J. A., Sweeney, S. A., Hayes, E. F., Carpenter, P. M. and Smith, M. L. (2007). Cannabinoid concentrations in hair from documented cannabis users. *Forensic Sci. Int.* **169**, 129–36.
82. Han, E., Chung, H. and Song, J. M. (2012). Segmental hair analysis for 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid and the patterns of cannabis use. *J. Anal. Toxicol.* **36**, 195–200.
83. Han, E., Yang, H., Seol, I., Park, Y., Lee, B. and Song, J. M. (2013). Segmental hair analysis and estimation of methamphetamine use pattern. *Int. J. Legal Med.* **127**, 405–11.
84. Namera, A., Konuma, K., Saito, T., Ota, S., Oikawa, H., Miyazaki, S., Urabe, S., Shiraishi, H. and Nagao, M. (2013). Simple segmental hair analysis for  $\alpha$ -pyrrolidinophenone-type designer drugs by MonoSpin extraction for evaluation of abuse history. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **942-943**, 15–20.

85. Martin, M., Muller, J. F., Turner, K., Duez, M. and Cirimele, V. (2012). Evidence of mephedrone chronic abuse through hair analysis using GC/MS. *Forensic Sci. Int.* **218**, 44–8.
86. De Kesel, P. M. M., Lambert, W. E. and Stove, C. P. (2014). O8: Metabolite-to-parent drug concentration ratios in hair to study metabolism? The case of CYP1A2 phenotyping. *Toxicol. Anal. Clin.* **26**, S8.
87. Fisichella, M., Steuer, A. E., Kraemer, T. and Baumgartner, M. R. (2014). O18: Chiral analysis of methadone and its main metabolite EDDP in hair: Incorporation depending on hair colour and metabolizer status. *Toxicol. Anal. Clin.* **26**, S12.
88. Gareri, J., Rao, C. and Koren, G. (2014). Examination of sex differences in fatty acid ethyl ester and ethyl glucuronide hair analysis. *Drug Test. Anal.* **6 Suppl 1**, 30–6.
89. Crunelle, C. L., Cappelle, D., Covaci, A., van Nuijs, A. L. N., Maudens, K. E., Sabbe, B., Dom, G., Michielsen, P., Yegles, M. and Neels, H. (2014). Hair ethyl glucuronide as a biomarker of alcohol consumption in alcohol-dependent patients: role of gender differences. *Drug Alcohol Depend.* **141**, 163–6.
90. Salomone, A., Pirro, V., Lombardo, T., Di Corcia, D., Pellegrino, S. and Vincenti, M. (2014). Interpretation of group-level factors from a large population dataset in the determination of ethyl glucuronide in hair. *Drug Test. Anal.* doi: 10.1002/dta.1697.
91. Høiseth, G., Morini, L., Ganss, R., Nordal, K. and Mørland, J. (2013). Higher levels of hair ethyl glucuronide in patients with decreased kidney function. *Alcohol. Clin. Exp. Res.* **37 Suppl 1**, E14–6.
92. Chan, J., Sauv e, B., Tokmakejian, S., Koren, G. and Van Uum, S. (2014). Measurement of cortisol and testosterone in hair of obese and non-obese human subjects. *Exp. Clin. Endocrinol. Diabetes* **122**, 356–62.
93. Veldhorst, M. A. B., Noppe, G., Jongejan, M. H. T. M., Kok, C. B. M., Mekic, S., Koper, J. W., van Rossum, E. F. C. and van den Akker, E. L. T. (2014). Increased scalp hair cortisol concentrations in obese children. *J. Clin. Endocrinol. Metab.* **99**, 285–90.
94. Gonz alez-Reimers, E., Mart n-Gonz alez, C., Galindo-Mart n, L., Aleman-Valls, R., Gonz alez-P erez, J. M., Jorge-Ripper, C., Elvira-Cabrera, O. and Quintero-Platt, G. (2014). Hair copper in normal individuals: relationship with body mass and dietary habits. *Trace Elem. Electrolytes* **31**, 67–72.
95. Gonzalez-Reimers, E., Mart n-Gonz alez, C., Galindo-Mart n, L., Aleman-Valls, M. R., Velasco-V azquez, J., Arnay-de-la-Rosa, M., P erez-Hern andez, O. and Luis, R. H. (2014). Lead, cadmium and zinc in hair samples: relationship with dietary habits and urban environment. *Biol. Trace Elem. Res.* **157**, 205–10.
96. Dussy, F., Carson, N., Hangartner, S. and Briellmann, T. (2014). Is one hair lock really representative? *Drug Test. Anal.* **6 Suppl 1**, 5–8.
97. LeBeau, M. a, Montgomery, M. a and Brewer, J. D. (2011). The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. *Forensic Sci. Int.* **210**, 110–6.



98. Han, E., Yang, W., Lee, J., Park, Y., Kim, E., Lim, M. and Chung, H. (2005). Correlation of methamphetamine results and concentrations between head, axillary, and pubic hair. *Forensic Sci. Int.* **147**, 21–4.
99. Kintz, P. (2012). Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption 2011. *Forensic Sci. Int.* **218**, 2.
100. Pirro, V., Di Corcia, D., Pellegrino, S., Vincenti, M., Sciutteri, B. and Salomone, A. (2011). A study of distribution of ethyl glucuronide in different keratin matrices. *Forensic Sci. Int.* **210**, 271–7.
101. Pianta, A., Liniger, B. and Baumgartner, M. R. (2013). Ethyl glucuronide in scalp and non-head hair: an intra-individual comparison. *Alcohol Alcohol.* **48**, 295–302.
102. Baumgartner, M. R., Binz, T. M. and Kraemer, T. (2014). O15: Determination of EtG in body hair samples for monitoring of teetotalism: A suitable alternative to scalp hair locks? *Toxicol. Anal. Clin.* **26**, S11.
103. Agius, R., Ferreira, L. M. and Yegles, M. (2012). Can ethyl glucuronide in hair be determined only in 3 cm hair strands? *Forensic Sci. Int.* **218**, 3–9.
104. Harland, D. P., Walls, R. J., Vernon, J. a, Dyer, J. M., Woods, J. L. and Bell, F. (2014). Three-dimensional architecture of macrofibrils in the human scalp hair cortex. *J. Struct. Biol.* **185**, 397–404.
105. Vincenti, M., Salomone, A., Gerace, E. and Pirro, V. (2013). Application of mass spectrometry to hair analysis for forensic toxicological investigations. *Mass Spectrom. Rev.* **32**, 312–32.
106. Vincenti, M., Salomone, A., Gerace, E. and Pirro, V. (2013). Role of LC-MS/MS in hair testing for the determination of common drugs of abuse and other psychoactive drugs. *Bioanal.* **5**, 1919–38.
107. Fisichella, M., Morini, L., Sempio, C. and Groppi, A. (2014). Validation of a multi-analyte LC-MS/MS method for screening and quantification of 87 psychoactive drugs and their metabolites in hair. *Anal. Bioanal. Chem.* **406**, 3497–506.
108. Montesano, C., Johansen, S. S. and Nielsen, M. K. K. (2014). Validation of a method for the targeted analysis of 96 drugs in hair by UPLC-MS/MS. *J. Pharm. Biomed. Anal.* **88**, 295–306.
109. Di Corcia, D., D’Urso, F., Gerace, E., Salomone, A. and Vincenti, M. (2012). Simultaneous determination in hair of multiclass drugs of abuse (including THC) by ultra-high performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **899**, 154–9.
110. Koster, R. a, Alffenaar, J.-W. C., Greijdanus, B., VanDernagel, J. E. L. and Uges, D. R. a. (2014). Fast and highly selective LC-MS/MS screening for THC and 16 other abused drugs and metabolites in human hair to monitor patients for drug abuse. *Ther. Drug Monit.* **36**, 234–43.

111. Fernández, M. D. M. R., Di Fazio, V., Wille, S. M. R., Kummer, N. and Samyn, N. (2014). 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.* **965**, 7–18.
112. Broecker, S., Herre, S., Wüst, B., Zweigenbaum, J. and Pragst, F. (2011). Development and practical application of a library of CID accurate mass spectra of more than 2,500 toxic compounds for systematic toxicological analysis by LC-QTOF-MS with data-dependent acquisition. *Anal. Bioanal. Chem.* **400**, 101–17.
113. Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., Bonner, R. and Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell. Proteomics* **11**, O111.016717.
114. Röst, H. L., Rosenberger, G., Navarro, P., Gillet, L., Miladinović, S. M., Schubert, O. T., Wolski, W., Collins, B. C., Malmström, J., Malmström, L., et al. (2014). OpenSWATH enables automated, targeted analysis of data-independent acquisition MS data. *Nat. Biotechnol.* **32**, 219–23.
115. Broecker, S., Herre, S. and Pragst, F. (2012). General unknown screening in hair by liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). *Forensic Sci. Int.* **218**, 68–81.
116. Nielsen, M. K. K., Johansen, S. S., Dalsgaard, P. W. and Linnet, K. (2010). Simultaneous screening and quantification of 52 common pharmaceuticals and drugs of abuse in hair using UPLC-TOF-MS. *Forensic Sci. Int.* **196**, 85–92.
117. Favretto, D., Vogliardi, S., Stocchero, G., Nalesso, A., Tucci, M. and Ferrara, S. D. (2011). 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* **1218**, 6583–95.
118. Miyaguchi, H. and Inoue, H. (2011). Determination of amphetamine-type stimulants, cocaine and ketamine in human hair by liquid chromatography/linear ion trap-Orbitrap hybrid mass spectrometry. *Analyst* **136**, 3503–11.
119. Moore, C., Rana, S., Coulter, C., Feyerherm, F. and Prest, H. (2006). Application of two-dimensional gas chromatography with electron capture chemical ionization mass spectrometry to the detection of 11-nor-Delta9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair. *J. Anal. Toxicol.* **30**, 171–7.
120. Conti, M., Tazzari, V., Bertona, M., Brambilla, M. and Brambilla, P. (2011). Surface-activated chemical ionization combined with electrospray ionization and mass spectrometry for the analysis of cannabinoids in biological samples. Part I: analysis of 11-nor-9-carboxytetrahydro-cannabinol. *Rapid Commun. Mass Spectrom.* **25**, 1552–8.

121. Mercolini, L., Mandrioli, R., Protti, M., Conti, M., Serpelloni, G. and Raggi, M. A. (2013). Monitoring of chronic Cannabis abuse: an LC-MS/MS method for hair analysis. *J. Pharm. Biomed. Anal.* **76**, 119–25.
122. Thieme, D., Sachs, H. and Uhl, M. (2014). Proof of cannabis administration by sensitive detection of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in hair using selective methylation and application of liquid chromatography- tandem and multistage mass spectrometry. *Drug Test. Anal.* **6**, 112–8.
123. Harrison, R. and Fu, S. (2014). A review of methodology for testing hair for cocaine. *J. Forensic Investig.* **2**, 1–8.
124. Schwaninger, A. E., Meyer, M. R. and Maurer, H. H. (2012). Chiral drug analysis using mass spectrometric detection relevant to research and practice in clinical and forensic toxicology. *J. Chromatogr. A* **1269**, 122–35.
125. Martins, L., Yegles, M., Chung, H. and Wennig, R. (2005). Simultaneous enantioselective determination of amphetamine and congeners in hair specimens by negative chemical ionization gas chromatography-mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **825**, 57–62.
126. Martins, L. F., Yegles, M., Chung, H. and Wennig, R. (2006). Sensitive, rapid and validated gas chromatography/negative ion chemical ionization-mass spectrometry assay including derivatisation with a novel chiral agent for the enantioselective quantification of amphetamine-type stimulants in hair. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **842**, 98–105.
127. Nishida, K., Itoh, S., Inoue, N., Kudo, K. and Ikeda, N. (2006). High-performance liquid chromatographic-mass spectrometric determination of methamphetamine and amphetamine enantiomers, desmethylselegiline and selegiline, in hair samples of long-term methamphetamine abusers or selegiline users. *J. Anal. Toxicol.* **30**, 232–7.
128. Kikura-Hanajiri, R., Kawamura, M., Miyajima, A., Sunouchi, M. and Goda, Y. (2011). Chiral analyses of dextromethorphan/levomethorphan and their metabolites in rat and human samples using LC-MS/MS. *Anal. Bioanal. Chem.* **400**, 165–74.
129. Kelly, T., Doble, P. and Dawson, M. (2005). Chiral analysis of methadone and its major metabolites (EDDP and EMDP) by liquid chromatography-mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **814**, 315–23.
130. Pirro, V., Valente, V., Oliveri, P., De Bernardis, A., Salomone, A. and Vincenti, M. (2011). Chemometric evaluation of nine alcohol biomarkers in a large population of clinically-classified subjects: pre-eminence of ethyl glucuronide concentration in hair for confirmatory classification. *Anal. Bioanal. Chem.* **401**, 2153–64.
131. Hoelzle, C., Scheufler, F., Uhl, M., Sachs, H. and Thieme, D. (2008). Application of discriminant analysis to differentiate between incorporation of cocaine and its congeners into hair and contamination. *Forensic Sci. Int.* **176**, 13–8.
132. Bossers, D. L. C. A. M. and Paul, D. R. (2014). Application of Bayesian theory to the reporting of results in alcohol hair testing. *Forensic Sci. Int.* **242**, e56–8.

133. Zhu, K. Y., Leung, K. W., Ting, A. K. L., Wong, Z. C. F., Fu, Q., Ng, W. Y. Y., Choi, R. C. Y., Dong, T. T. X., Wang, T., Lau, D. T. W., et al. (2011). The establishment of a highly sensitive method in detecting ketamine and norketamine simultaneously in human hairs by HPLC-Chip-MS/MS. *Forensic Sci. Int.* **208**, 53–8.
134. Zhu, K. Y., Leung, K. W., Ting, A. K. L., Wong, Z. C. F., Ng, W. Y. Y., Choi, R. C. Y., Dong, T. T. X., Wang, T., Lau, D. T. W. and Tsim, K. W. K. (2012). Microfluidic chip based nano liquid chromatography coupled to tandem mass spectrometry for the determination of abused drugs and metabolites in human hair. *Anal. Bioanal. Chem.* **402**, 2805–15.
135. Chen, Y., Li, M., Fu, L., Hou, X. and Jiang, X. (2014). Simultaneous determination of trace cadmium and lead in single human hair by tungsten electrothermal vaporization-flame atomic fluorescence spectrometry. *Microchem. J.* **114**, 182–186.
136. Dressler, V. L., Pozebon, D., Mesko, M. F., Matusch, A., Kumtabtim, U., Wu, B. and Sabine Becker, J. (2010). Biomonitoring of essential and toxic metals in single hair using on-line solution-based calibration in laser ablation inductively coupled plasma mass spectrometry. *Talanta* **82**, 1770–7.
137. Thieme, D. and Sachs, H. (2007). Examination of a long-term clozapine administration by high resolution segmental hair analysis. *Forensic Sci. Int.* **166**, 110–4.
138. Miki, A., Katagi, M., Kamata, T., Zaitso, K., Tatsuno, M., Nakanishi, T., Tsuchihashi, H., Takubo, T. and Suzuki, K. (2011). MALDI-TOF and MALDI-FTICR imaging mass spectrometry of methamphetamine incorporated into hair. *J. Mass Spectrom.* **46**, 411–6.
139. Miki, A., Katagi, M., Shima, N., Kamata, H., Tatsuno, M., Nakanishi, T., Tsuchihashi, H., Takubo, T. and Suzuki, K. (2011). Imaging of methamphetamine incorporated into hair by MALDI-TOF mass spectrometry. *Forensic Toxicol.* **29**, 111–6.
140. Porta, T., Grivet, C., Kraemer, T., Varesio, E. and Hopfgartner, G. (2011). Single hair cocaine consumption monitoring by mass spectrometric imaging. *Anal. Chem.* **83**, 4266–72.
141. Vogliardi, S., Favretto, D., Frison, G., Ferrara, S. D., Seraglia, R. and Traldi, P. (2009). A fast screening MALDI method for the detection of cocaine and its metabolites in hair. *J. Mass Spectrom.* **44**, 18–24.
142. Vogliardi, S., Favretto, D., Frison, G., Maietti, S., Viel, G., Seraglia, R., Traldi, P. and Ferrara, S. D. (2010). Validation of a fast screening method for the detection of cocaine in hair by MALDI-MS. *Anal. Bioanal. Chem.* **396**, 2435–40.
143. Musshoff, F., Arrey, T. and Strupat, K. (2013). Determination of cocaine, cocaine metabolites and cannabinoids in single hairs by MALDI Fourier transform mass spectrometry--preliminary results. *Drug Test. Anal.* **5**, 361–5.
144. Shen, M., Xiang, P., Shi, Y., Pu, H., Yan, H. and Shen, B. (2014). Mass imaging of ketamine in a single scalp hair by MALDI-FTMS. *Anal. Bioanal. Chem.* **406**, 4611–6.
145. Poetzsch, M., Baumgartner, M. R., Steuer, A. E. and Kraemer, T. (2014). Segmental hair analysis for differentiation of tilidine intake from external contamination using LC-ESI-MS/MS and MALDI-MS/MS imaging. *Drug Test. Anal.* doi: 10.1002/dta.1674.

146. Jackson, A. U., Garcia-Reyes, J. F., Harper, J. D., Wiley, J. S., Molina-Díaz, A., Ouyang, Z. and Cooks, R. G. (2010). Analysis of drugs of abuse in biofluids by low temperature plasma (LTP) ionization mass spectrometry. *Analyst* **135**, 927–33.
147. Nielen, M. W. F., Nijrolder, a W. J. M., Hooijerink, H. and Stolker, a a M. (2011). Feasibility of desorption electrospray ionization mass spectrometry for rapid screening of anabolic steroid esters in hair. *Anal. Chim. Acta* **700**, 63–9.
148. Duvivier, W. F., van Beek, T. a, Pennings, E. J. M. and Nielen, M. W. F. (2014). Rapid analysis of  $\Delta$ -9-tetrahydrocannabinol in hair using direct analysis in real time ambient ionization orbitrap mass spectrometry. *Rapid Commun. Mass Spectrom.* **28**, 682–90.