Chapter 10
End User Commentary on Recent Technological Developments in MALDI-MSI Based Hair Analysis

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In recent years, significant technical developments in hair analysis have been made. One of the most significant ones is the introduction of MALDI-MSI in hair analysis. Since the publication of Vogliardi et al. in 2009 on the fast detection of cocaine and metabolites in hair, research articles using MALDI analysis on hair boomed. There are several reasons for the great interest of the forensic hair analysis community in this technique, particularly for the easy and fast acquisition of detailed timeline information on multiple compounds incorporated in hair. Moreover, this can be achieved on a single hair sample, which was never shown before in chemical compound analysis (drugs and medicines).

As nicely described in this chapter by Flinders et al., incorporation of compounds in hair is a complicated process that makes forensic interpretation of drug analysis results very difficult. Not only external contamination is a difficult issue, on which a lot of research is already published, but also the different growth phases, that play a role in the incorporation rate, makes it even more complicated. Nevertheless, hair can be the only evidence left in a forensic case. Thanks to the stability (only matrix left after putrefaction) and potential timeline information of medication/drug use/abuse, hair is often indispensable evidence in court. The greatest challenges for forensic experts in current hair analysis (using decontamination, extraction and GC- or LC-mass spectrometry) are:

- The high amount of hair (10–100 mg) necessary for analysis
- Segmentation of hair necessary to make a timeline (highest detail possible per month)
- The laborious decontamination and extraction procedure
- The differentiation between contamination and user
- The determination of relevant cut-offs that differentiate a sporadic from chronic user (for example ethyl glucuronide in alcohol consumption)

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S. Francese (ed.), *Emerging Technologies for the Analysis of Forensic Traces*, Advanced Sciences and Technologies for Security Applications,
https://doi.org/10.1007/978-3-030-20542-3_10

Additionally, despite these technical and interpretation challenges, it is very difficult to explain in an intelligible way, hair analysis results in court. As a forensic expert, explaining the possible effect of decontamination procedures (wash-in and wash-out effects), the use of cut-offs and the interpretation of time segments, all based on technical information such as chromatograms and mass spectra, to a non-scientifically trained jury, is challenging.

It is exactly thanks to the overcoming of many of these challenges that Mass Spectrometry Imaging (MSI) became popular in hair research:

- Analysis became possible on one single hair
- Complete timeline is visible in a single analysis without segmentation
- Sample preparation is rather easy, cheap and fast
- Direct visualization of contamination versus use is possible thanks to longitudinal sectioning
- Molecular images are very easy to understand for a court jury

The chapter gives a nice overview of the key publications and current applications of MALDI-MSI in hair analysis. From my perspective, I would like to stress two major developments within the last years that can tackle some critical discussion points within the hair analysis society: sample preparation and direct visualization of contamination versus abuse/consumption.

Sample preparation

Figure 9.3 in the Chap. 9 shows that very limited sample preparation is required for MALDI-MSI. This makes it possible to gather very fast and detailed information without segmenting and extensive extraction procedures. Even decontamination procedures might become unnecessary since longitudinal cuts can 'open-up' the hair for gathering internal information. Thus, discussions about wash-in and wash-out effects of current decontamination procedures can be avoided in the future, making conveying the evidence more straightforward and 'stronger' in court. The longitudinal sectioning procedure and the accompanying bespoke device is described in detail within this chapter. However, it would be helpful in routine forensic practice if the sectioning tool became commercially available. Therefore considering sample preparation in MSI, the crucial step from lab development to routine practice with regards to practice standardization still needs to be made.

Direct visualization of contamination versus abuse/consumption

As indicated in the chapter, issues regarding the possible wash-in and wash-out effect of the current decontamination procedures are reported in the literature. This makes interpretation of the detection of several compounds very complicated. Parent compounds-to-metabolites ratios have been suggested to solve this problem and it is current practice. Moreover, the analysis of the washing procedure becomes indispensable as an evidence. Nevertheless, for some compounds, this remains a 'grey zone' rather than a black-white story and it sometimes makes the credibility of the current hair analysis technique for court rather low. Despite the fact that both (extraction and MSI) methods can have the same scientific value, it is my opinion that the

possibility of using MSI to directly visualize the difference between contamination or use in a photograph-like picture (and thus much easier to understand) will be considered as 'stronger' evidence for a jury.

The same applies to the direct visualization of a drug intake timeline. Instead of comparing different extracts from different segments (including possible extraction variations on the different segments), MSI makes it possible to show intake in one single molecular image, making interpretation much easier.

Thanks to the direct, more comprehensive information MSI can give, this technique has several advantages over the standard segmenting-extracting procedures today. Nevertheless, despite these great advantages, there are still some issues to solve before the technique can be translated from the lab to routine forensic practice:

- **Sensitivity** for a much broader range of drugs need to be evaluated
- **Quantitative** determination is necessary in order to give the correct interpretation regarding sporadic versus chronic user
- Sample preparation and analysis should be **validated**
- More **real cases samples** need to be investigated to technique's capabilities and versatility

Sensitivity Sensitivity of the technique for different compounds should be further investigated. As described in the chapter, Beasley et al. showed that derivatization might help to improve the sensitivity necessary for low concentration hair samples. Nevertheless, this can depend on the technique used. Therefore, standardisation and validation of sample preparation as well as MSI analysis is necessary in order to be used in forensic practice. Moreover, it can be expected that not all compounds can be visualized in hair. Understanding whether this depends on the lack of incorporation or ionization efficiency of the compound is crucial to eventually establish routine protocols. Only when MSI can be as sensitive as the standard extraction-analysis method and thus can visualize forensically relevant concentrations, it will become a very appealing technique for routine labs. This will most likely be related with the MALDI-matrix used (beyond ionization efficiency of the analyte) and standardization and validation of this aspect is also indispensable.

Quantitation Quantitation is necessary in order to draw correct conclusions regarding sporadic or chronic user. Therefore, standardisation and validation is necessary. Internal standards are indispensable for correct quantification. As described in the chapter, several issues arise regarding the use of standards. It would be interesting to study the difference between the spiked dilutions (isotopic labelled standards in matrix solution is a possibility) on the hair matrix and the prepared matrix matched hair. Is there a difference? This is important in order to make a standardized protocol and control samples. Standards of compounds in different hair colours can be interesting in standardizing the method. Considering hair colour differences, it might be a great advantage of MSI to link the chemical hair colour determinants (melanin content as shown by Rosen et al.) with the compound concentration. Poetzsch already showed that the ionization of compounds is independent of the hair colour, which is crucial information in validating MALDI methods for hair analysis. Nevertheless,

some questions on the quantitation method still need to be tackled before a standardized routine method is ready for forensic investigation. But it can be expected that MALDI will have some interesting advantages over standard methods used today thanks to the ability to reveal the full chemical profile, including hair colour, in one single longitudinal cut hair analysis.

Validation In order for MALDI MSI analysis to be implemented in forensic investigations, quality controls for compound determination and quantification in hair is indispensable. As stated in the chapter, matrix matched standards can be considered as the most ideal solution as quality control. This, combined with isotopically labelled standards mixed in the matrix solution may lead to a fully validated and controlled quantitative MALDI-MSI method. Nevertheless, another issue could arise within the adoption of MSI: since only one or just a few hairs can be used in MSI, it should be investigated how many hairs need to be imaged in order to be representative. This is certainly important taking into account different growth stages. An example of the importance of this question became already noticeable in the paper of Shen et al. showing differences in ketamine content in 4 imaged hairs of the same person. Of course the same argument can be used for standard extraction analysis, but due to the high amount of hairs in the extraction process, the mean is considered to be representative.

The incorporation can differ depending on the growth stage. Therefore if it is possible to determine the growth state through chemical biomarkers, it might be possible to link the growth stage with the concentration and the interpretation.

Case proven In the chapter, it is stated that one of the advantages of MSI is the detailed timeline that becomes available and this is, indeed, a very positive aspect to the use of the technology. Nevertheless, it should be taken into consideration that, while sampling (cutting), it is already described that a small time shift is introduced. Thus, the question can be raised whether it is relevant to measure with a detail of 22 min. Therefore, time frame experiments such as presented by Kamata et al. (for methoxyphenamine) and Poetzsch et al. and Shima et al. (zolpidem) are important to be able to draw the correct conclusions from imaging experiments and not overrate the timing possibilities.

Moreover, interindividual incorporation and growth rate differences can be expected. Although these remarks are important in the overall interpretation of hair analysis results, MSI can introduce an added value since differences can directly be related to the optical image and chemical difference determined by hair colour, colouring agent and other cosmetic products. As already previously stated, thanks to this full chemical profile, concentration differences in certain time areas can directly be linked to hair treatments that also can be visualized. This new possibility should be investigated further in real case samples.

This brings me to a more general remark on the translation of MSI from research to court: basic research is necessary in order to understand full capabilities and limitations. However, the technique will only be accepted by the forensic community

when more real case samples will be analyzed using MSI. In ideal circumstances, both techniques (extraction and MSI) should be compared on real forensic case samples.

New possibility One of the new possibilities that comes along with MSI is the investigation of a much broader compound range. In the same single analysis run, also lifestyle markers, as reported by Nakanishi et al., becomes possible. This opens new forensic areas such as profiling suspects from one single hair found on the crime scene. Future research will indicate whether this new possibility will make it to court cases.

Analysis cost and time A final, but not in the least important, is the analysis cost and time. Thanks to the continuous instrumental developments, MSI has already significantly improved in the last few years in terms of sensitivity, mass accuracy, mass resolution and structural identification. Together with better matrix application devices, these improvements have led to more reproducible results (beneficial for validation purposes) and to faster sample preparation, meaning lower analysis cost. Since in most countries, forensic investigation budget is very limited and time pressure is very high, analysis cost and time are an often-underestimated requirement of research labs that try to translate their methods to routine practice. The highest cost, that currently hinders MSI to become a routine method in forensic labs, is the equipment cost itself. When imaging equipment becomes more affordable, I am convinced that MALDI-MSI applied to hair will become routine practice since the sample preparation and running costs are rather low compared to the standard laborious extraction techniques. Moreover, new possibilities and major advantages of MALDI-MSI are already shown, making it a very appealing technique that will be translated from research technique into routine forensic hair analysis.