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Simona Francese *Editor*

Emerging Technologies for the Analysis of Forensic Traces

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Emerging Technologies for the Analysis of Forensic Traces

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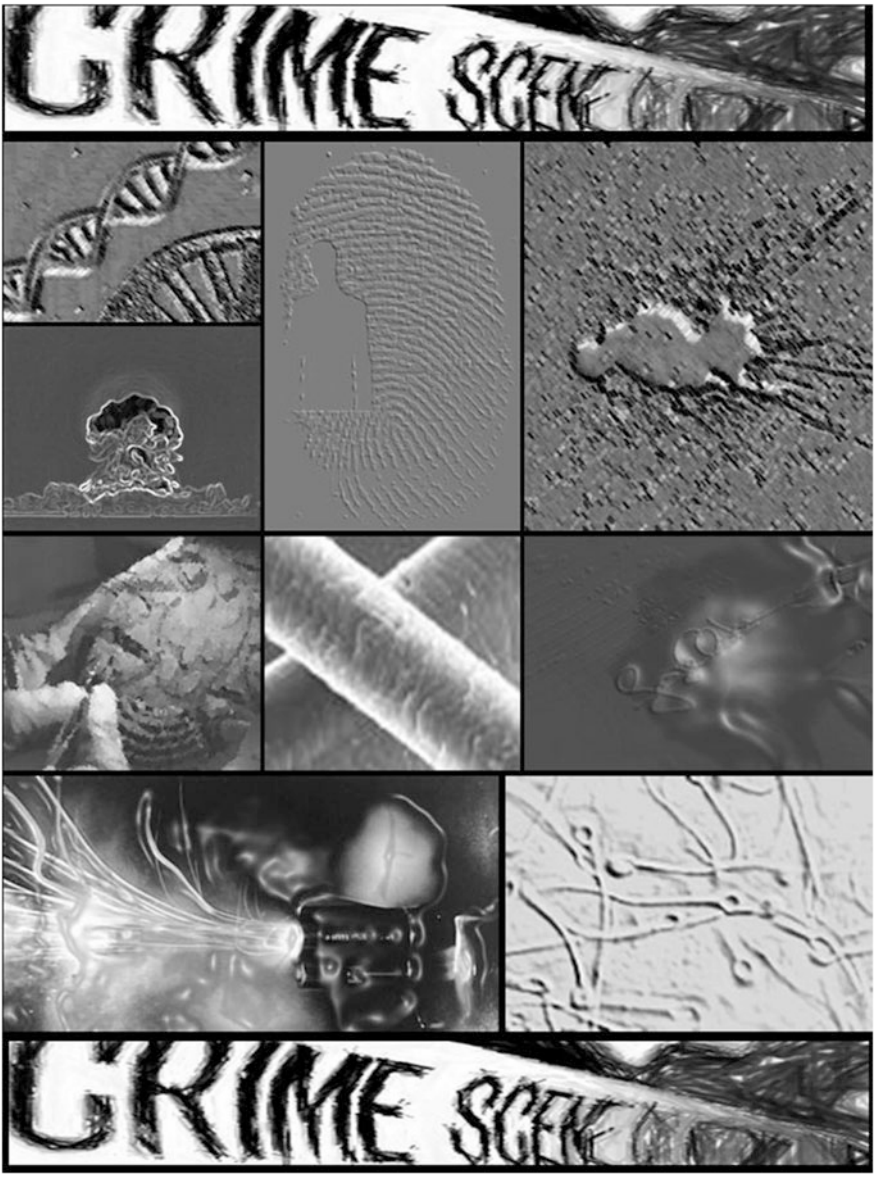

CRIME SCENE
CRIME SCENE

To my father, for being the rock and the lighthouse of my life.

To my mother, for being an inexhaustible source of love and support.

To my sister, for being a constant inspiration and the only example I know of true strength, courage, sacrifice and altruism.

To my husband, for never trying to contain my passion for research and for silently supporting me through illness and in all those mad work sleepless nights and frequent travels.

To my daughter Grace, for teaching me how to still see the world with enchanted eyes and for making me special—a mother

Preface

The work of detectives in gathering intelligence through conventional investigations is paramount to criminal apprehension and to inform judicial debates. However, through the years, it has become clear that the role of forensic science for the analysis of trace evidence can often be crucial to steer investigations and make court cases more robust.

The opportunity for forensic science to contribute to narrow down the pool of suspects and provide scientific evidence to prove/disprove the defendant's statement can have a tremendous impact on savings to the public purse and on speeding up investigations by minimising human resources. Most importantly, more and better informed judicial debates would contribute to avoid or minimise miscarriage of justice.

It is perhaps for these reasons that in the last ten years, forensic science has progressed enormously; a tremendous amount of technological developments in chemistry and analytical chemistry has been channelled into contributing to additional, more robust and informative intelligence recovered from trace evidence.

Advances in nanotechnology, molecular and spectroscopic analysis have shown to have a remarkable impact on the quality and quantity of the intelligence that can be recovered from a range of trace evidence. The vast majority of these methods were successfully applied in laboratory settings, demonstrating high potential for operational casework. Whilst some technologies show a great promise to be translated to the field, some others have also started to be occasionally employed in an operational context, an example of which is the use of MALDI Mass Spectrometry Imaging to reconstruct fingerprint molecular images as well as providing profiling information around the owner of the mark. Soil analysis (Chap. 17) stands out as it is fully operational and used in both investigations and courtroom settings. With regard to soil analysis, in line with the many novel methods which are being developed in mainstream forensic trace evidence, as shown in all the other chapters, a similar range of analytical methods could be applied, including physical,

chemical and biological methods. However, because soils are so diverse and complex and geoforensics is an infrequently used trace evidence type, this chapter has covered the legal aspects of soil analysis, most of which can relate to the other evidence types described elsewhere in the book.

This book intends to offer a survey of the state of the art of emerging technologies applied to a range of forensic trace evidence including fingermarks, bodily fluids, hair, gunshot residues, explosives, ink and questioned documents.

Importantly, whilst not losing sight of the description of the technical aspects of each methodology, huge efforts have gone into the adoption of a controlled vocabulary enabling access to a wider range of readers.

As eventually these technologies and associated methodologies will have to pass the scrutiny of law enforcement agencies, at the end of each chapter, end users have provided a commentary discussing the potential for operational deployment of these emerging processes. This novel feature mitigates potential "academic hypes" as well as adding another dimension: *what is required to take an emerging technology from the laboratory to the courtroom* from a practitioner's perspective. This collaboration and the adoption of a controlled vocabulary pose the bases to build a dissemination and communication bridge between academia and end users/practitioners, thus facilitating the transition from laboratory technologies to fieldwork. Given the peculiarity of Chap. 17, two end users have been invited to separately provide a commentary as they offer two different perspectives, one from a law enforcement viewpoint (Scottish Police Authority) and one from a "forensic provider" perspective (Netherlands Forensic Institute).

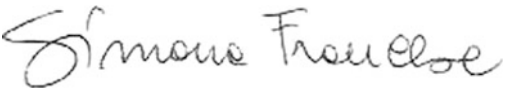
Any means to facilitate translational research is timely, and this is especially true in the field of forensic science where progress is often hindered by lack of *appropriate* dissemination of scientific advancements as well as of fundamental communication between academia and end users.

Lastly, the issue of lack of a framework serving evidence-based interpretation has been brought up in many chapters of this book. In different ways and through different examples, the danger of falling into the trap of overlooking activity-level information by heavily placing focus on source-level information has been reported. More and more documented miscarriages of justice have been documented because of this.

I believe that new emerging technologies need to develop within the above understanding with funding and projects dedicated to expand capabilities from source-to-activity-level information provision.

It is my expectation that this book will be of interest to forensic scientists, chemists, biologists and analytical chemists with an interest in forensic science; the book intends to deepen knowledge and understanding in both the science being covered and with respect to the expectations on the law requirements to fulfil, enabling the technique being developed to become operational.

The book intends to be also useful to practitioners, policy-makers and scientific officers managing forensic and analytical portfolios within UK research councils as well as worldwide funding bodies through updating their knowledge of current and state-of-the-art scientific developments.



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Simona Francese

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Abbreviations

AAR	Aspartic acid racemisation
ABS	Acrylonitrile butadiene styrene
ACE-V	Analysis, comparison, evaluation and verification
AFM	Atomic force microscopy
AIF	All-ion fragmentation
ALS	Alternate light sources
ANPR	Automatic number plate recognition
ASTM	American Standard and Testing Materials
ATR	Attenuated total reflectance
AUC	Area under the curve
BCT	Big City Trial
BY40	Basic Yellow 40
BZE	Benzoylecgonine
CCRC	Criminal Cases Review Commission
CDR	Cartridge discharge residue
CE	Capillary electrophoresis
CHCA	α-cyano-4-hydroxycinnamic acid
CLSM	Confocal laser scanning microscopy
CMV	Capillary microextraction of volatiles
COCE	Cocaethylene
COPFS	Crown Office and the Procurator Fiscal Services
CPR	Civil Procedure Rules
CPS	Crown Prosecution Service
CrimPR	Criminal Procedure Rules
CSI	Crime scene investigators
CWL	Chromatic white light sensors
DAPNe	Direct analyte-probed nano-extraction
DART	Direct analysis in real time
DBD	Dielectric barrier discharge
DESI	Desorption electrospray ionisation

DMDNB	Dimethyl dinitrobutane
DNT	Dinitrotoluene
DPA	Diphenylamine
EBSD	Electron backscattered diffraction detector
EDNAP	European DNA profiling group
EDX	Energy-dispersive X-ray spectrometry
EGDN	Ethylene glycol dinitrate
ENFSI APST	European Network of Forensic Science Institutes–Animal Plant and Soil Traces
ENFSI	European Network of Forensic Science Institutes
EPA	Environmental Protection Agency
EPR	Electron paramagnetic resonance
ESM	Environmental scanning microscopy
FAR	False alarm rate
FBI	Federal Bureau of Investigation
FDR	Firearms discharge residue
FLE	Filtered light examination
FMPTS	2-fluoro-1-methylpyridinium toluenesulfonate
FPR	False positive rate
FRS	Forensic Science Regulator
FTICR	Fourier-transform ion cyclotron resonance
FTIR	Fourier-transform infrared spectroscopy
FWHM	Full width at half maximum
GC	Gas chromatography
GO	Graphene oxide
GSR	Gunshot residues
HEXIL	Hexanitrodiphenylamine
HMF	Heavy metal free
HMTD	Hexamethylene triperoxide diamine
HNS	Hexanitrostilbene
HP	Hydrogen peroxide
HPLC	High-performance liquid chromatography
(HP)TLC	High-performance thin-layer chromatography
HRMS	High-resolution mass spectrometry
HSI	Hyperspectral imaging
IBA	Ion beam analysis
IC	Ion chromatography
ICP-AES	Inductively coupled plasma–atomic emission spectrometry
IE	Improvised explosive
IEDs	Improvised explosive devices
IMS	Ion-mobility spectrometry
INAA	Instrumental neutron activation analysis
IS	Internal standard
IUGS-IFG	International Union of Geological Sciences–Initiative on Forensic Geology

LA ICP MS	Laser ablation inductively coupled plasma mass spectrometry
LA	Laser ablation
LAGs	Liquids, aerosols and gels
LC	Liquid chromatography
LCTF	Liquid crystal tunable filter
LDI	Laser desorption ionisation
LESA	Liquid extraction surface analysis
LIBS	Laser-induced breakdown spectroscopy
LOD	Limit of detection
LPAS	Laser photoacoustic spectroscopy
LTP	Low-temperature plasma
m/z	Mass-to-charge
MALDI	Matrix-assisted desorption ionisation mass spectrometry
MC	Methylcentralite
MEKC	Micellar electrokinetic capillary chromatography
MIR	Mid-infrared
MPE	Maximum permissible energy
MPI	Laser multiphoton ionisation
mRNA	Messenger RNA
MS	Mass spectrometry
MSI	Mass spectrometry imaging
MSP	Microspectrophotometry
NCTC	National Counterterrorism Center
NDNAD	National DNA Database
NFI	Netherlands Forensic Institute
NG	Nitroglycerine
NIR	Near-infrared
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NQ	Nitroguanidine
ODS	Octadecylsilane
OGSR	Organic gunshot residue
OP	Optical profilometry
PCR	Polymerase chain reaction
PCR	Principal component regression
PDMS	Polydimethylsiloxane
PETN	Pentaerythritol tetranitrate
PIGE	Proton-induced gamma emission
PIXE	Particle-induced X-ray emission
PMDE	Pendant mercury drop electrode
PPS	Public Prosecution Service
PSA	Prostate-specific antigen
PSPME	Planar solid-phase microextraction
QD	Quantum dot
RADEX	Raman detection of explosives

RDX	1,3,5-Trinitro-1,3,5-triazinane
ROC	Receiver operating characteristic
ROI	Region of interest
RT-PCR	Reverse transcriptase–polymerase chain reaction
SEM	Scanning electron microscope
SERS	Surface-enhanced Raman spectroscopy
SIMS	Secondary ion mass spectrometry
SNP	Single-nucleotide polymorphism
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
STANDEX	Standoff detection of explosives
STRs	Short tandem repeats
SWGGSR	Scientific Working Group for Gunshot Residue
TAGs	Triacylglycerides
TATP	Triacetone triperoxide
TD	Thermal desorption
TEA	Thermal energy analyser
TEM	Transmission electron microscopy
TENAC	Tetranitro-acridone
THC	Δ^9-tetrahydrocannabinol
TLC	Thin-layer chromatography
TNP	Trinitrophenol
TNT	2,4,6-Trinitrotoluene
TNTAB	Triazido-trinitrobenzene
ToF	Time of flight
TPR	True positive rate
TRLs	Technology readiness levels
UKAS	United Kingdom Accreditation Service
UPLC	Ultra-performance liquid chromatography
VIP	Variable ionisation potential
VSC	Video spectral comparator
XRD	X-ray diffractometry
XRF	X-ray fluorescence

Chapter 1
Mass Spectrometry Methods for the Recovery of Forensic Intelligence from Fingermarks

Melanie J. Bailey and Catia Costa

Abstract Mass spectrometry is a method of identifying molecules within a sample, based on a characteristic mass to charge ratio. Over the last decades, it has become possible to use mass spectrometry to obtain high resolution molecular images of surfaces. In this chapter, we will show how mass spectrometry techniques can be used to obtain high quality images of fingerprints, determine their placement compared with other traces (for example overlapping fingerprints or inks) and determine their chemical make-up for offender profiling purposes.

1.1 Introduction

Fingerprints have contributed crucial information to police investigations for the last 150 years and have helped to solve countless crimes around the globe. Despite the rise of DNA evidence, in the UK, the fingerprint database still contains a greater number of records and is also the most commonly used evidence type by most police forces [1, 2].

There is some debate over the exact definition of the terms fingerprint and fingermark in the forensic community [2]. In general terms, a fingermark is the trace that is left at the crime scene, through contact with a surface by a suspect. Typically, a fingermark is invisible to the naked eye and must be chemically developed in order to recover the characteristic ridge details. The various development methods that are used by police forces for the recovery of fingermarks have been comprehensively documented by the UK Home Office Centre for Applied Science and Technology and collaborators [3, 4]. Once the fingermark has been developed and photographed, the ridge patterns are compared to an inked fingerprint, given by a known donor. In this chapter, a fingermark refers to a mark deposited in an uncontrolled manner by

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an unknown donor, whereas a fingerprint is deposited in a controlled manner by a known donor.

A fingerprint consists of material that is excreted by a donor through the eccrine glands, which are located under the skin of the fingers [5]. Eccrine sweat is comprised of water, amino acids, salts and urea. As well as containing eccrine sweat, a fingerprint from unwashed hands will contain material that a donor has touched [6]. This normally includes sebum, which is excreted from the sebaceous glands found on the face and in the hair. Sebum is composed of squalene, wax esters, triglycerides and phospholipids. A fingerprint also contains exogenous substances (i.e. substances present due to contact rather than excretion), for example hair products and cosmetics [7].

Over the last decades, a great number of studies have investigated the chemistry of fingerprints in order to gain a deeper understanding of their chemical make-up. These efforts are summarized in a number of review papers [2, 8, 9]. This research can support the development of fingermark enhancement techniques and be used to gain additional intelligence around the donor. These studies have been carried out using a variety of analytical techniques based on spectroscopy and mass spectrometry. In this chapter, we will consider only the recent advances made by mass spectrometry techniques. It will be discussed how mass spectrometry techniques can be used to obtain high quality images of fingerprints, determine their placement compared with other traces (for example overlapping fingerprints or inks) and determine their chemical make-up for offender profiling purposes.

Key application areas—The efficacy of a particular development reagent to recover fingermarks depends on multiple factors. Fingermarks are highly variable in chemical composition, and there are many contaminants a suspect may have touched that may interfere with the development process. Additionally, the ageing of chemical species deposited within a fingerprint depends on the nature of the deposition surface and the environment a fingerprint has been exposed to after deposition [3]. This means that under particular circumstances, the recovery rate of fingermarks can be very low. In this chapter, it is illustrated how mass spectrometry can be used to improve the recovery of fingermarks.

The ability of a forensic tool to provide a link between a questioned source (i.e. a fingermark) and a known source (a fingerprint) is known as *source level information* [10]. This has been used successfully for over 150 years to place suspects at crime scenes, but gives no information about the circumstances of deposition. Acting only at the source level limits the probative value of many investigations—for example, what if an offender had legitimate access to the crime scene? Over the past decade, there has been considerable interest from law enforcement agencies in expanding the scope of fingerprint evidence to give not only source level but also "activity level" information. In this chapter it will be illustrated how, using mass spectrometry, locating chemical traces within a fingerprint can be used to establish some of the circumstances surrounding its deposition.

For a fingermark found at a crime scene, the ridge detail provides useful information only if there is a match to a suspect or on a fingerprint database. Much recent research has explored the possibility of using a fingermark for offender profiling, to

provide investigative leads. In this chapter we will explore the progress that has been made in using chemical profiling of a fingerprint to obtain donor-specific information, for example their gender, lifestyle or medical history.

What is mass spectrometry?—The process of mass spectrometry (MS) involves generating and measuring ions (molecules with a positive or negative charge) from a sample [11]. There are many methods for generating ions (for example by firing a laser, ion beam or solvent at the sample) as it will be illustrated later in this chapter. In a mass analyzer, ions are separated according to their mass to charge (*m/z*) ratio. This produces a spectrum of peaks corresponding to different ions, (Fig. 1.1) allowing putative identification of a peak to be made based on its *m/z* value. To provide greater

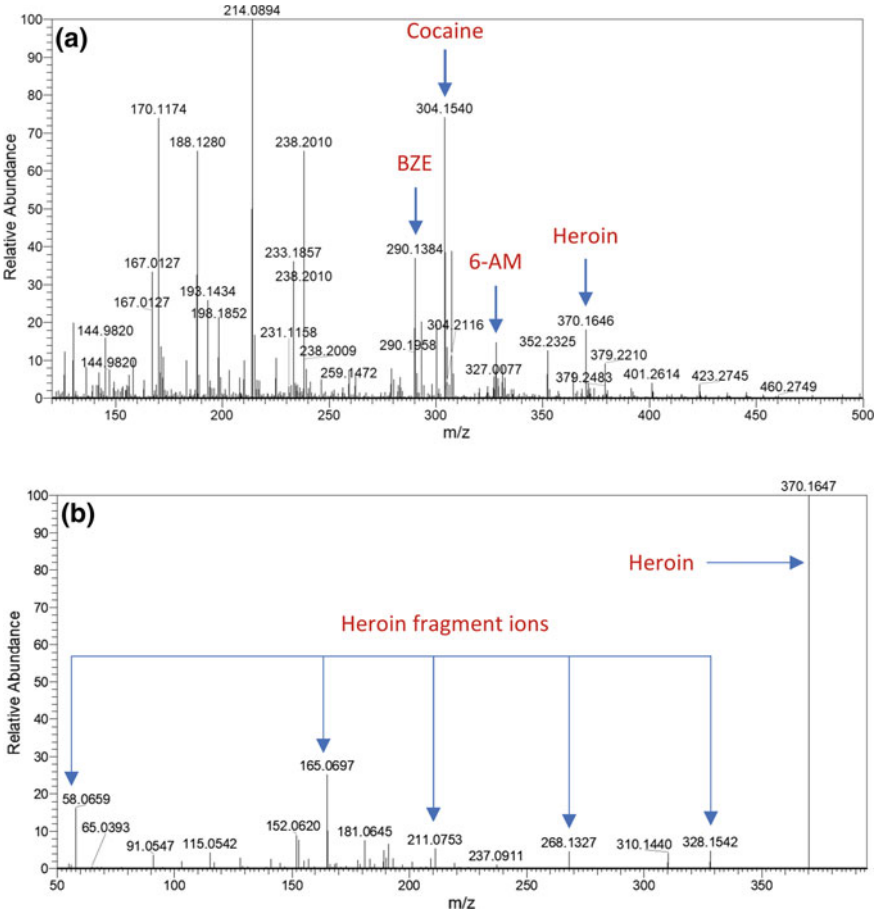

Fig. 1.1 **a** Overlaid mass spectra of cocaine, heroin, benzoylecgonine (BZE) and 6-monoacetylmorphine (6-AM) standards, generated using liquid chromatography mass spectrometry (LC MS); **b** tandem mass spectrum, showing the characteristic fragment ions (indicated by arrows) of heroin

confidence on the identity of a molecule, tandem mass spectrometry is employed to "break" a parent ion into fragment ions. These *m/z* fragments are often characteristic of a specific molecule thus can be used to confirm identity of a molecule.

Chromatography—Chromatographic techniques such as gas and liquid chromatography are traditionally used to separate sample constituents prior to mass spectrometry [12, 13]. The ionization of analytes can be suppressed or enhanced by other species present in the sample, and the effect of chromatography is to reduce these matrix interferences. Chromatography assists therefore in providing both quantitative analysis as well as enhanced sensitivity (the latter in case of ion suppression with MS alone).

In a chromatography system, a chromatography column is packed with a material (μm in particles diameter) known as a stationary phase. The sample is injected into a mobile phase (in liquid chromatography (LC), a solvent; in gas chromatography (GC) a gas) before it is passed on to the column. Separation of analytes is determined by the affinity of these chemicals to the stationary phase. The greater the affinity, the longer it takes for the analytes to pass through the column. The time at which the compound elutes (leaves the column) is called the retention time. The retention time is characteristic for a given analyte under specific conditions (such as type of column, mobile phase and flow rate). In forensic analysis, the characteristic retention time (alongside the mass to charge ratio of its parent and fragment ions) is used to identify a specific compound. In LC-MS, analytes eluting from the column must be ionized before introduction into the mass spectrometer. For LC-MS, this is normally carried out by electrospray ionization, where the eluate emerging from the column is pumped through a charged, heated metal capillary; for GC-MS, ionization is typically achieved via electron impact. It is then possible to produce an extracted ion chromatogram (XIC), where the time dependency of a particular ion signal is plotted. As an output example, XIC's corresponding to drugs and a lipid extracted from tissue is given in Fig. 1.2.

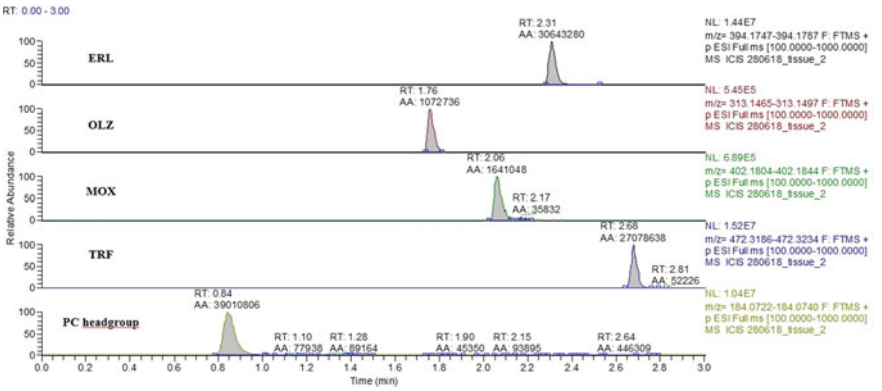

Fig. 1.2 Example extracted ion chromatograms, showing the separation of four drugs and a lipid headgroup using liquid chromatography mass spectrometry

Mass analyzers—There are many different types of mass spectrometry instrumentation, from portable devices that can be carried in a car or backpack [14] to high performance laboratory-based systems. At the time of writing this chapter, portable mass spectrometry systems do not yet appear to have been successfully applied to fingermark analysis. That said, the capability of portable mass spectrometers are rapidly improving and may form the basis of future systems for use in forensics [15].

Of the laboratory-based mass analyzers, there are two types that are typically used in forensic laboratories for toxicology: triple quadrupoles and Orbitrap. Detailed descriptions of the mode of operation of these detectors can be found elsewhere [11]. Triple quadrupoles will be found in most forensic toxicology laboratories and are favoured because of their high sensitivity and quantitative capability. However, the quadrupole mass analysers have low resolution (typically ≈1000 FWHH) and low ion selectivity (unit resolution). It is for this reason that toxicologists typically use chromatography to separate sample constituents prior to mass spectrometry, to increase selectivity, as well as tandem mass spectrometry. A limitation of triple quadrupole instrument is the difficulty in carrying out untargeted analysis—i.e. to screen a sample for unknown compounds. More recently, Orbitrap mass spectrometers are being adopted by many forensic toxicology laboratories for high resolution mass spectrometry (HR-MS, mass resolution up to ≈500,000 FWHH) and the possibility to employ all ion fragmentation (AIF) for untargeted analysis [16].

1.2 Information Gained from Traditional Mass Spectrometry Approaches

There have been a number of initial scoping exercises that have explored the chemistry of fingerprints under different conditions and as a function of age. These generally employed the conventional techniques of liquid chromatography mass spectrometry (LC-MS) or gas chromatography mass spectrometry (GC-MS) [17–19]. It should be noted that these standard chromatography techniques require the entire fingerprint to be extracted from a surface prior to analysis. This is not only time-consuming, but also may be unfeasible in forensic casework where it is undesirable to destroy the only fingermark recovered. However, gaining an understanding of basic fingerprint chemistry using established techniques has been important in obtaining ground knowledge on how fingerprint composition can vary between individuals and over time, and is therefore discussed in this section. The readiness of these techniques for operational work is discussed in the final part of this chapter and summarized in Table 1.1, at the end of the chapter.

Fingerprint ageing—Age determination of forensic traces is a highly sought after forensic capability and is discussed in Chap. 2. Obtaining a better understanding of fingerprint ageing will not only help to establish a model for establishing the time of deposition, but enables the forensic community to optimize reagents that are used to develop them. A great number of studies have employed mass spectrometry

Table 1.1 Grading scale of operational readiness per application and per technique

Application	Technique	SIMS	MeV SIMS	MALDI	DESI	LESA	DAPNe	paper spray	LC-MS
Enhanced imaging		4	1	2					
Drug detection for toxicology				1	2	2		4	5
Drug detection for intelligence		2		5	1	2	1	3	3
Fingerprint ageing		1		1					1
Fingerprint and ink deposition order		4	1						
Sex determination				1	1				
Overlapping fingerprints				1					
Fingerprints in blood				3					
Explosive detection				1		1	1	3	3

Key	
1	Testing on ideal substrate or on selected samples
2	Tested under operationally relevant condidtions
3	Tested on a large number of samples
4	Tested in pseudo-operational trials
5	Already deployed operationally

with chromatography to gain information on chemical changes of fingerprints as a function of age, and these are summarized by Cadd et al. [2]. Through these studies it has been shown that fatty acids, squalene, cholesterol, and triglyceride decrease over time after a fingerprint has been deposited. More recently, work by de Puit et al. has shown that protein degradation in fingerprints can be monitored as a function of time using LC-MS [20]. However, these studies have also highlighted the complexity of the issue, with the ageing process of smaller molecules showing a dependency on the substrate material [18], donor [19] and exposure to heat [21], light [18, 22], humidity and vacuum [17]. The dependency of the fingerprint proteome on these factors is yet to be assessed.

Donor profiling—A number of studies have attempted to use the chemistry of a fingerprint to classify a donor. For example Girod et al. determined fatty acids, squalene, cholesterol and triglycerides in the fingerprints of a large number of donors using GC-MS [23]. de Puit et al. [24] determined the amino acid profiles of donors using LC-MS and found high variability between the fingerprints of different donors, indicating an ability to distinguish the fingerprints of donors based on their chemical profile. Whilst the methodology itself has been validated for repeatability [25], there is some considerable way to go to determine whether donor classification can be used operationally. In particular, it is not known whether the chemistry of a reference fingerprint (deposited by a donor under controlled conditions, on a different day/time to the committed crime) would bear any resemblance to the chemistry of a fingermark deposited at a crime scene (following contact with various substances, ageing after deposition and application of a visualization agent). Additionally, previous studies have observed considerable intra donor variability in fingerprint composition [26, 27] and therefore tying down a chemical profile of a fingerprint to specific donor is unlikely to be straightforward. Nonetheless, these studies form an important basis for future work.

Drug intake—A number of studies have adapted and employed LC-MS methods from forensic toxicology on fingerprint samples to determine drug use [28–30]. Methadone [31], lorazepam [32], cocaine and heroin [33] have been detected in the fingerprints of drug users using LC-MS. Our group has also detected codeine

and morphine in fingerprints above environmental levels using LC-MS, as shown in Fig. 1.3. Ismail et al. [33] showed that even though cocaine is frequently detected in the fingerprints of non-drug users, it is possible to set a cut-off value to distinguish environmental exposure from cocaine use. Additionally, cocaine and heroin can be transferred from a drug user to a non-drug user by hand shaking. The transferred drugs can be subsequently removed by handwashing. This means that provided a donor washes their hands prior to donating a fingerprint, cocaine is unlikely to be detected as a result of either environmental exposure or secondary transfer from a drug user. These results are now being used to support commercial drug testing in the UK .

A recent study also by Ismail et al. [34] has demonstrated that adherence to a treatment regime can also be established from a fingerprint. Whilst the intended application was medical compliance testing, this finding may have future forensic value in determining whether a suspect was adhering to their treatment regime at the time of committing an offence. It was found that for patients taking isoniazid (the medication used to treat tuberculosis), either the parent drug or the metabolite was detected using LC-MS in almost all cases. By collecting samples from patients as they completed their treatment, it was found that a fingerprint gives an ideal window of detection for monitoring compliance, with all traces of the drug disappearing 2–3 days after the medication is ceased. This may have implications in the field of forensic fingermark analysis if, for example, it is suspected that patient may not have

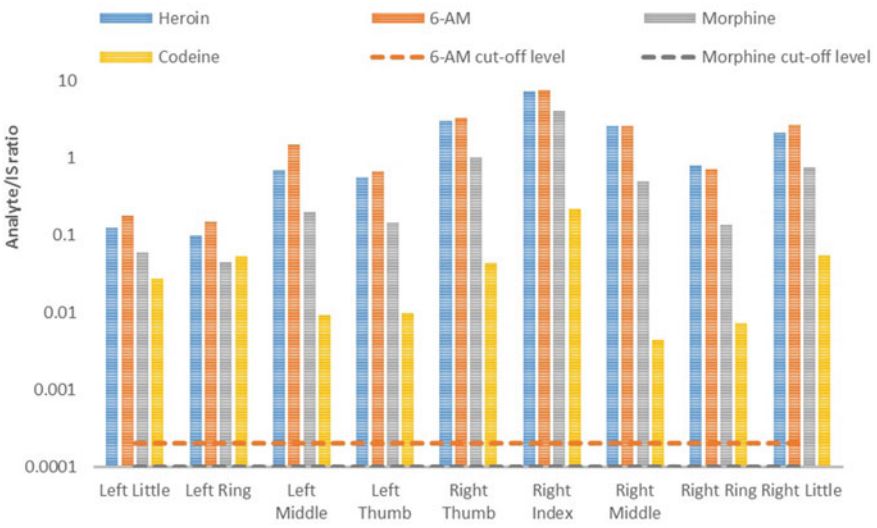

Fig. 1.3 Signal intensity of analyte relative to an internal standard (IS) of codeine, morphine, heroin and 6-acetylmorphine detected in fingerprints of a drug user detected using paper spray mass spectrometry. A "cut-off" level, based on the fingerprints of non-drug users, is given for comparison [87]

been taking their medication at a time of committing an offence, or to assist with donor profiling.

It is important to note that the studies discussed in this section focused on the use of fingerprints donated (under controlled conditions) for drug testing (forensic toxicology) rather than on (uncontrolled) fingermark samples. The fingerprint studies use donors with cleaned hands, clean substrates and ageing under controlled conditions. This is a very different situation to receiving a fingermark from an unknown suspect under uncontrolled conditions which has been aged in an unknown manner. Additionally, when a fingermark is recovered, it is necessary to apply a chemical developer in order to visualize it. The studies in this section have not considered the chemical changes that undoubtedly take place when a fingermark is visualized. Therefore at present, chromatographic analysis of fingerprints can, and are, being used support drug testing, but further development would be needed before these techniques could be applied operationally for the analysis of fingermarks.

A drawback of chromatography techniques for drug testing is the time that is required to extract fingerprint samples from their substrate, which adds considerably to the cost of analysis. In the next section, we report on the use of direct mass spectrometry approaches to rapidly analyse the chemical composition of fingerprints.

1.3 Rapid, Non-imaging Techniques—Paper Spray Mass Spectrometry

Paper spray mass spectrometry is a technique developed by Purdue University in 2010 and has now been commercialized for dried blood spot analysis [35–37]. Paper spray mass spectrometry requires only a simple ionization source—a sample is collected onto a piece of paper that is cut into a triangle, as shown in Fig. 1.4. Solvent is applied to the paper to dissolve and extract analytes, and when a high voltage is applied to the back end of the triangle, extracted analytes are swept into the inlet of the mass spectrometer. Costa et al. analysed 159 patient samples and found that cocaine administration could be determined from a single fingerprint in a 2 min method using paper spray mass spectrometry [38]. Additionally, fingerprints could be visualized with silver nitrate prior to detection of cocaine via paper spray mass spectrometry.

As described in relation to LC-MS, it should be noted that analysis using paper spray mass spectrometry is most suited to drug testing, rather than analysis of fingermarks. However, the work provides some important observations on the interpretation of drugs detected in a fingerprint, and demonstrates the compatibility of fingerprint visualization followed by drug testing.

A considerable challenge for fingerprint drug testing is the possibility that the drug residue arises from drug contact, rather than drug use. As mentioned previously, the work of Ismail et al. [33] has shown that cocaine and heroin use can be distinguished from environmental levels and/or secondary transfer by setting an appropriate cut-off

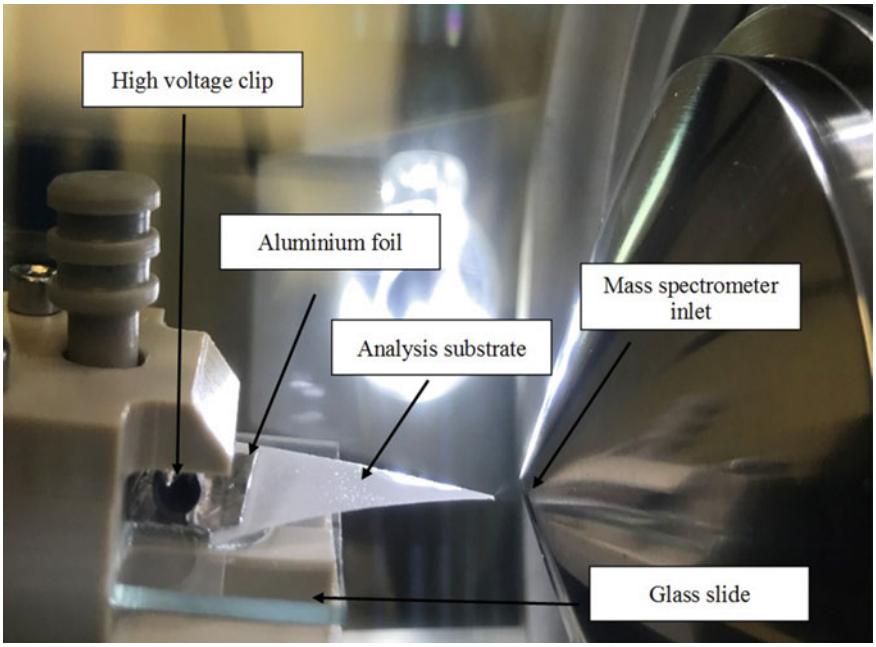

Fig. 1.4 Photograph showing paper spray mass spectrometry. A fingerprint can be placed on the paper triangle and a voltage is applied to the clip at the back end of the paper. Here, a glass slide and aluminium foil are used to shield the paper from carryover effects. Solvent is applied to the paper and a Taylor cone forms at the end of the tip, which sweeps analytes from the paper into the mass spectrometer

level. However this still leaves open the possibility that the donor simply touched a substance but was not under the influence, hence the potential importance of detecting drug metabolites, and this is currently being explored in our group.

For either paper spray or LC-MS analysis, although the ridge details of the fingerprint can be visualized prior to analysis, the techniques give no information on whether the analytes detected actually reside in the fingermarks. This is a considerable drawback to using chromatography techniques operationally on uncontrolled samples (fingermarks) because the method gives no spatial information; a fingerprint deposited on top of a trace (e.g. paint, explosives, drugs, blood) would look exactly the same as a fingerprint containing the same trace and then deposited on a surface. In these situations, an imaging technique may be preferred, and the next section will report on mass spectrometry imaging.

1.4 Mass Spectrometry Imaging

In mass spectrometry imaging, a mass spectrum is recorded from each pixel of a sample [39, 40]. This allows separate images of each *m/z* peak to be generated, as shown in Fig. 1.5. Ions must be generated from a sample using an ionization source that provides a beam of photons or particles that can be focused into a small spot. There are three main types of commercially available ion sources for mass spectrometry imaging and these employ ions (secondary ion mass spectrometry) [41], lasers (matrix assisted laser desorption ionisation) [42] and solvent spray (desorption electrospray ionization) [43] to remove and ionise material from a sample.

In contrast to chromatography applications, where either triple quadrupoles or Orbitrap detectors are preferred, time of flight (ToF) mass analyzers are a popular choice for mass imaging spectrometry applications. This is because a ToF can be used to rapidly detect thousands of mass peaks simultaneously with higher mass resolution ~45,000 FWHM (albeit quantitative capabilities are much less straightforward). ToFs are generally faster than Orbitraps, and this enables faster image acquisition.

1.4.1 Secondary Ion Mass Spectrometry (SIMS)

Secondary ion mass spectrometry (SIMS) is widely utilized by the semiconductor industry for high sensitivity characterization of contaminants on surfaces [41]. The fact that fingerprints could be visualized by SIMS has been known by the community for decades, but thought as more of an inconvenience than an advantage [44].

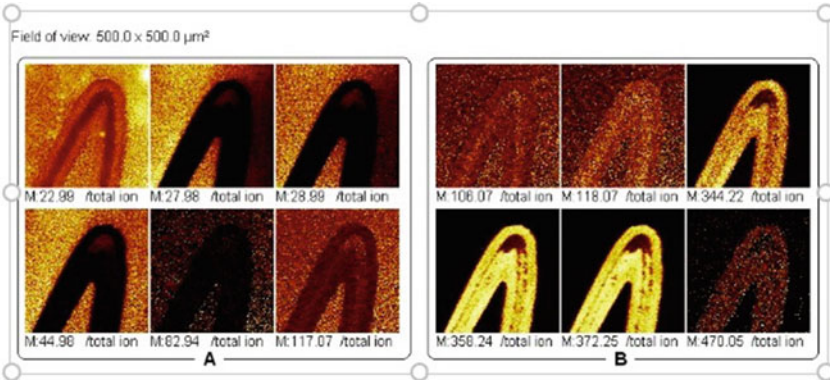

Fig. 1.5 Positive ion images of **a** ions and molecular ion fragments originating from a silicon wafer substrate and **b** ions and molecular ion fragments originating from a Staedtler 4300M black ballpoint pen ink deposited on the silicon wafer substrate, obtained using secondary ion mass spectrometry, adapted from Nick Bright, Ph.D. Thesis, submitted October 2012

Recently, a growing number of groups internationally have started to exploit the technique for fingerprint analysis [4, 26, 45–48].

Secondary ion mass spectrometry uses a primary ion beam (usually Bi^+ or Ga^+) to irradiate the surface of a sample. The ion beam causes erosion (sputtering) of molecules, which are then normally and subsequently fragmented. The ion beam can be focussed to sub-micron spot size and scanned across a sample, which offers the possibility of providing high resolution images. For a fingerprint, this means that it is possible to see third level detail (Fig. 1.6). Information is typically collected from the first few monolayers of material. Szynkowska et al. have shown that fingerprints can be imaged on glass, metal surfaces and paper using ToF-SIMS [49].

It has been shown by Szynkowska et al. that drug residue (after contact with amphetamine drugs) could be imaged in undeveloped fingerprints deposited on metal and glass substrates using ToF-SIMS [50]. In contrast, Bailey et al. 2015 failed to detect the (presumably) lower levels of cocaine in the fingerprints of drug users using the same technique [51]. In 2017, Cai et al. successfully demonstrated that graphene oxide could be used to enhance ToF-SIMS images of high molecular weight substances [46]. In Fig. 1.7, the molecular ion image of an antibiotic in a fingerprint is shown. It is as yet unknown whether the increase in sensitivity afforded by the use of graphene oxide would be sufficient for the imaging of fingerprints from drug users, and whether the use of graphene oxide is compatible with standard fingerprint visualization processes or other substrates.

Determining the deposition order of a fingermark and ink on a forensic specimen could be of virtue in cases where a suspect claims to have no knowledge of the contents of the document—for example having only loaded the printer. Conventional development procedures only give two dimensional details, and cannot prove whether a fingerprint was deposited before or after the ink was deposited. In 2014, Attard Montalto et al. showed that the deposition order of a fingerprint and laser printed ink

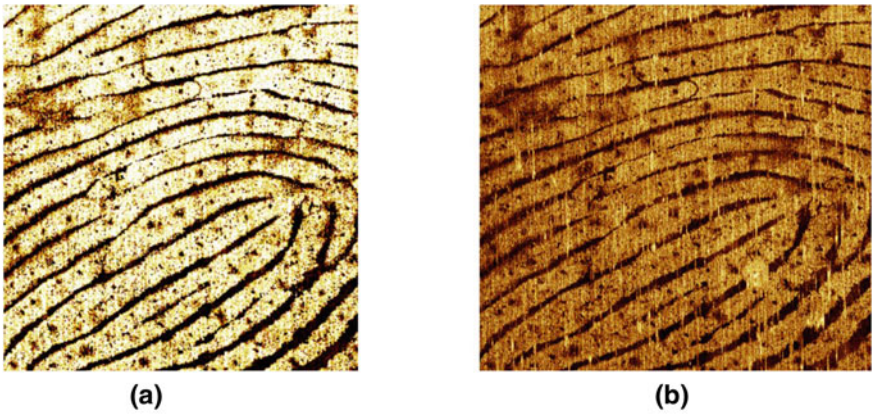

Fig. 1.6 ToF-SIMS images of **a** potassium (K) and **b** sodium (Na) in a fingerprint from a female donor deposited on brass substrate showing third level detail

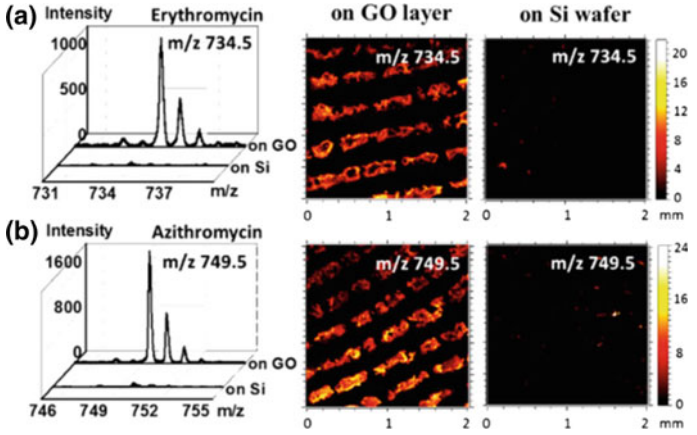

Fig. 1.7 SIMS images of fingerprints spiked with antibiotics with (left) and without (right) the presence of Graphene Oxide (GO) nanoparticles. Reprinted with permission from Cai et al. [46]. Copyright 2017 American Chemical Society

on a document could be successfully predicted using ToF-SIMS [48]. 156 known samples and 51 blind-test samples were analyzed, including 37 samples developed by either ninhydrin swabbing, ninhydrin spraying or iodine fuming. A depletion series of 14 fingerprints were prepared, with ink deposited in various ink-fingermark sequences on different days. These were analyzed at different times between 1 and 421 days after fingermark deposition. For inkjet or ballpoint pen inks, the deposition order was difficult to predict after the fingermark had been developed. However when a laser jet ink was used, in the instance where the fingerprint is deposited on top of the ink (Fig. 1.8a), the ridges continue over the ink lines, even after development with ninhydrin. In contrast, when the fingerprint is deposited below the ink, the ridges become discontinuous. In this blind inter-laboratory study, the deposition or was determined correctly 100% of the time, although it should be noted that only 4 samples were analyzed here.

Muramoto et al. found that the migration of lipids within a fingerprint deposited on a silicon wafer could be reliably predicted using ToF-SIMS imaging [52]. Whilst a silicon wafer substrate has limited operational relevance, Sisco et al. had previously found that the ability to spatially map fingerprint constituents was not affected by standard powder dusting techniques [47]. It would be necessary to test a range of substrates, donors, ageing and development conditions before operational deployment could take place.

A particular strength of the SIMS technique appears to be its ability to "develop" weak or poorly visualized fingermarks. In 2013, our group provided anecdotal evidence that SIMS could be used to enhance the development of fingerprints deposited on a variety of different substrates (Aluminum foil, a hand grenade handle a glass slide) and after different ageing conditions (burial in sea water, soil immersion) compared with development by conventional reagents [53]. Our more recent work

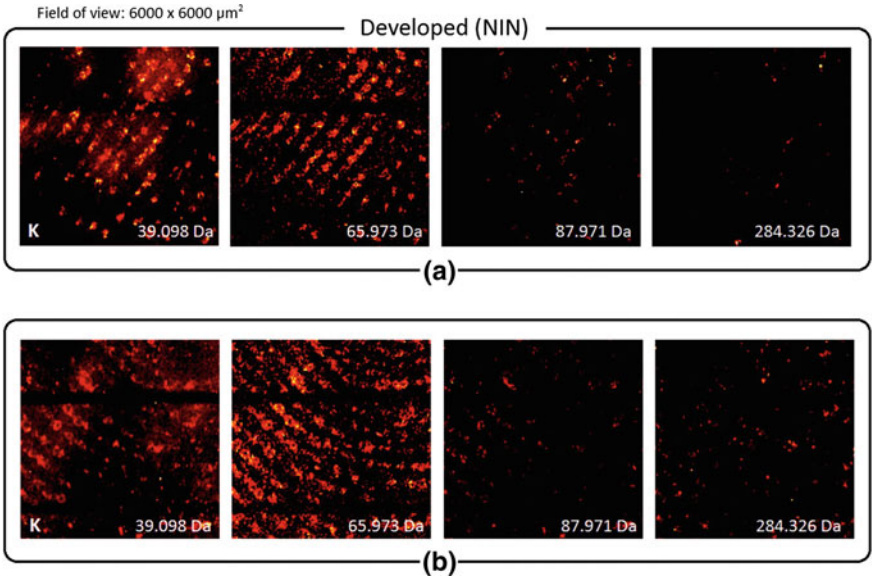

Fig. 1.8 ToF-SIMS chemical images of fingerprints deposited **a** on top of ink and **b** below ink on paper following ninhydrin development. Reproduced from Attard-Montalto et al. [48], published by the Royal Society of Chemistry

has shown that the technique performs well on marks that are partially developed by ninhydrin, cyanoacrylate, disulfur dinitride, vacuum metal deposition on paper, brass, plastic or steel substrates (Costa et al., in preparation). Figure 1.9 shows how a partially developed fingerprint can be enhanced using ToF-SIMS imaging; here an optical image (a) and (b) a SIMS image from the area marked by a red square of a fingerprint from female donor on ABS plastic substrate after contact with aqueous substance (bleach) and developed with cyanoacrylate + BY40 are shown. Similarly, Fig. 1.9c, d report an optical image and overlay of signals from sodium and an organic ion (originating from the fingerprint) and Cu (from the substrate) from area marked in red square for a fingerprint contaminated with bleach and deposited on brass then developed with S_2N_2. The generation of these 5×5 mm^2 images takes 25 min and larger images can be generated at correspondingly higher acquisition time. More recently, researchers from University of Nottingham have demonstrated the ability to image fingerprints with SIMS where samples prepared under the same conditions could not be developed using cyanoacrylate [54]. Work is in progress to understand more fully the conditions under which SIMS can provide enhanced images.

A drawback of using the SIMS technique for imaging a fingermark is that many systems operate under ultra-high vacuum conditions. Therefore samples must be introduced several hours before analysis takes place, and preferably cut down to a small size. MeV-SIMS (which uses an MeV ion beam to generate secondary ions) is a technique under development for ambient pressure mass spectrometry imaging. It has been the subject of an International Atomic Energy Agency Coordinated Research

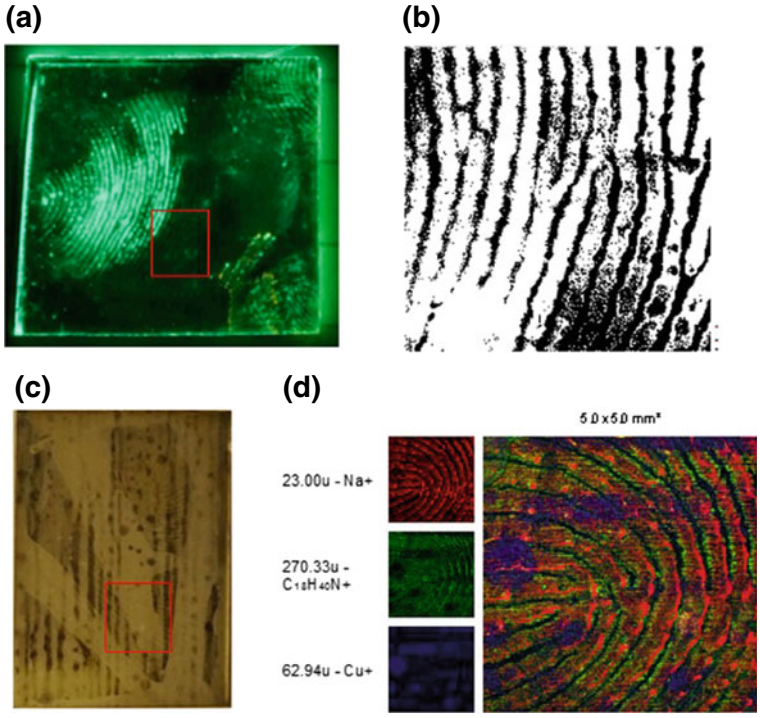

Fig. 1.9 **a** Optical image; **b** SIMS image from area marked by red square of a fingerprint from female donor on plastic substrate after contact with aqueous substance (bleach) and developed with cyanoacrylate + basic yellow 40; **c** optical image and **d** overlay of sodium (Na), organic ion at 270.33 and copper (Cu) signals from area marked in red square for fingerprint contaminated with bleach and deposited on brass then developed with S_2N_2

programme, with many national facilities internationally researching the technique [55]. Our group has shown previously that fingerprints and inks can be imaged using MeV-SIMS [26, 56], but the technique requires considerable development before it can be deployed operationally. In future however, this may provide a solution to the low throughput of SIMS systems.

1.4.2 Laser Desorption Ionisation (LDI) Approaches

Laser Desorption Ionisation approaches refer to methods in which the molecules are desorbed from the surface using a laser. Matrix assisted laser desorption ionisation (MALDI), the most widely adopted LDI approach, offers imaging of intact molecules, unlike SIMS, which tends to sputter fragments or atomic species. In contrast, MALDI typically operates at a lower spatial resolution of around 10–100 μm. MALDI is used widely by the biomedical community for imaging of biomolecules

in tissue samples, and more recently, driven by the team of Francese et al., has been used operationally for the analysis of fingermarks [57].

In MALDI, analytes are desorbed from the surface of a sample by firing a UV laser at the sample. The sample must first be coated with a low molecular weight, non-volatile organic molecule, known as a matrix, which enables absorption of the laser energy in the UV region. In imaging mode, a laser is fired at a raster of points defined by x, y, coordinates. A mass spectrum is obtained from each *x*, *y* location to generate 2D molecular maps of the detected ions.

Bradshaw et al. [58] used MALDI imaging to demonstrate that overlapping fingerprints could be resolved, either by imaging a single contaminant peak (e.g. caffeine from a coffee drinker) or through endogenous components. Figure 1.10 shows how overlapping groomed fingerprints (fingerprints collected by touching the face prior to deposition) can be resolved from one another through multivariate analysis of the MALDI image data though separation has been also achieved with ungroomed and natural fingerprints.

Bradshaw et al. [59] used MALDI imaging to demonstrate that direct contact with condom lubricants could be detected in fingermarks, even when the fingermarks were aged for 1–3 months. As shown in Fig. 1.11, multiple ion species relating to polyethylene glycol (PEG) ions found in a condom lubricant "Condomi max love" could be imaged on a fingerprint deposited on an aluminum foil. Although research is needed to explore the interpretive value of this result (in terms of persistence of lubricants on the hands), this capability has been exploited in casework (unpublished) and does highlight the ability of MALDI to detect and image contact residues in fingerprints [60, 61]. In particular, the use of imaging to show a spatial correspondence between the fingerprint ridges and a compound of interest may serve to strengthen the hypothesis that the substance was touched by a donor.

One of the particular strengths of MALDI is the ability to image molecules in a wide mass range (600–16,000 Da) as well as larger molecules. To this effect, Bradshaw et al. [62] and Deininger et al. [63] have shown that haem and haemoglobin and other blood specific proteins can be imaged in blood fingerprints. Moreover, the technique was capable of distinguishing equine and bovine blood from human blood. Whilst the methodology described by this group has as yet only been applied a limited range of substrates and development processes so far (albeit on very old specimens too), it shows promise for determining the presence of human blood in a fingerprint sample [62–64].

In order to deploy MALDI operationally, various approaches have been considered to assure compatibility with operational procedures. To facilitate compatibility with a range of substrates, Ferguson et al. developed the dry-wet method, in which a matrix (α-cyano-4 hydroxycinnamic acid) is dusted onto the fingermark to allow visualisation of the ridges [65]. The fingermark is then removed from the substrate via tape-lift, and the lifted mark is sprayed with a solvent mist to allow dissolution of the analytes in the fingerprint ridges and subsequent MALDI analysis. This then allows MALDI imaging to take place for fingerprints initially deposited on any substrate from which fingerprints can be effectively tape-lifted. However, at this stage it is unlikely that the dry-wet method will be used as a primary enhancement technique.

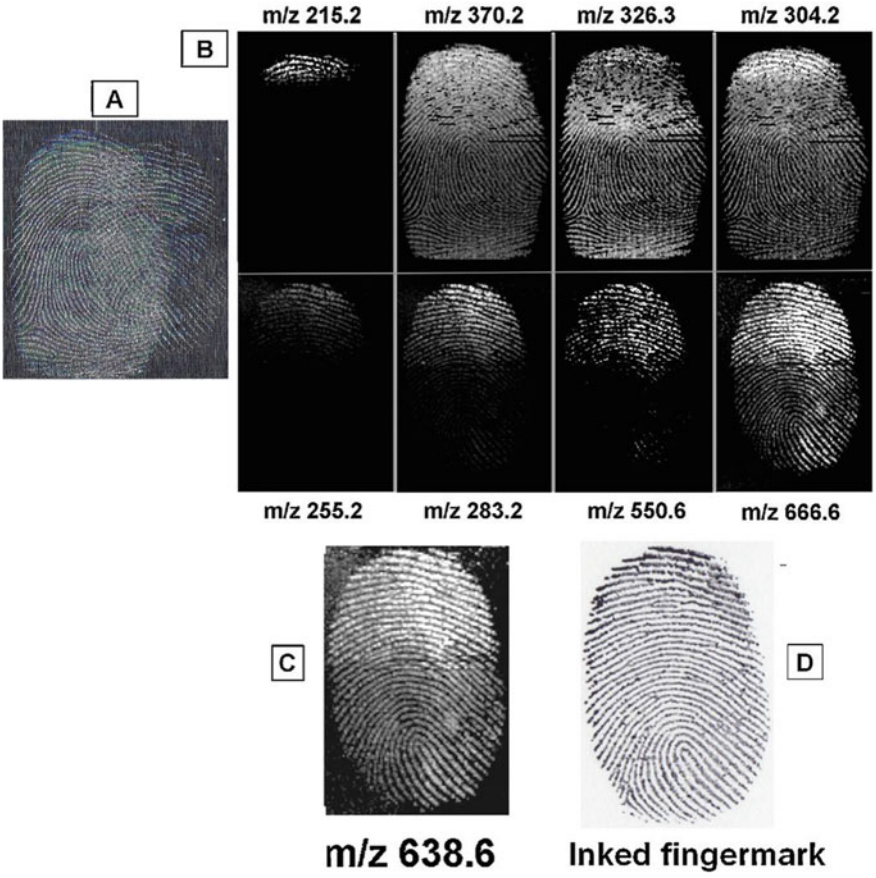

Fig. 1.10 MALDI MSI separation of overlapping groomed fingermarks. Panel **a** shows the optical image of the dusted overlapping fingermarks deposited by two different donors. Mass image separation of the two fingermarks is demonstrated through the selection of several ion signals characteristic of each fingermark (**b**). The image of the ion at *m/z* 638.6 (donor 1, **c**) was selected showing good ridge pattern clarity even in the area that was originally overlapped. A scan image of a fingerprint deposited after rolling the same donor's fingertip on an ink pad is also displayed for comparison purposes (**d**). Reprinted from Bradshaw et al. [58], with permission from Elsevier

For this reason, Francese's group has extensively (and successfully) worked towards making MALDI MSI compatible with the prior application of CSI and crime lab enhancement techniques on a range of difference surfaces with both in situ (including porous surfaces such as papers) and tape lifting analysis [64, 66, 67].

Alternatively, American and Canadian researchers have been employing the deposition of silver onto the fingerprint, in place of the MALDI matrix [68–70]. This LDI method is known as silver-assisted LDI. This allows fingermarks to be imaged directly on non-conductive surfaces without the need for tape lifting. This is presumably, particularly advantageous for porous surfaces (such as paper and cardboard) where the

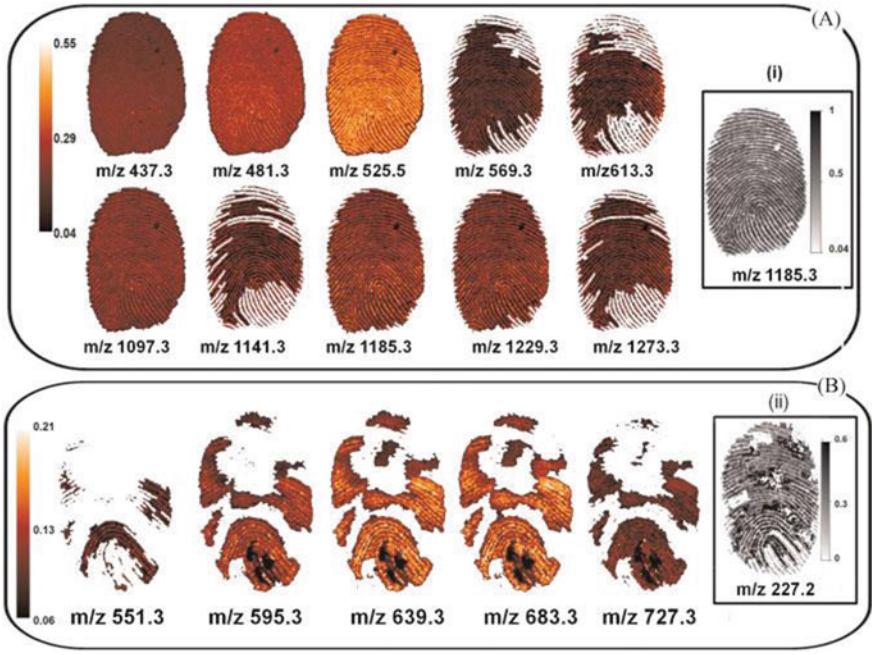

Fig. 1.11 MALDI MS images of aged condom contaminated (*Condomi max love*) fingermarks. The lubricant is still detected after ageing the fingermark for 1 month. Ridge details are still clearly visible and can be further enhanced with image software as the insert (i) shows for the ion at *m/z* 1185.3. Reprinted from Bradshaw et al. [59], with permission from Wiley

fingermark diffuses into the surface and cannot be effectively lifted (though paper can be analyzed directly by MALDI without the need of any lifting). The deposition of silver onto the fingerprint reduces matrix interferences in the low mass range so a useful approach for the detection of low mass compounds. Additionally, Lauzon et al. were able to also show a certain degree of compatibility as previously enhanced bloodied fingermarks could be enhanced using silver-assisted LDI [69], as shown in Fig. 1.12.

Kaplan-Sandquist et al. [71] showed that MALDI imaging could be used to image pharmaceutical drugs and explosives after contact with powder residues. Drug signals were not detected following contact with a pill, or broken tablet. A further observation was that after powder dusting, spreading of the drug beyond the ridges was observed. In a later publication, the same authors [72] compared a number of development methods (MALDI matrix, powder dusting, powder dusting followed by tape lift and cyanoacrylate fuming) for this application and found analyte spreading to be less problematic. This illustrates a requirement to train both the personnel who develop the fingermark and those who apply the matrix to ensure that neither process compromises the spatial integrity of the fingermark or associated contaminants, given that Francese's group has not observed analyte migration and matrix spreading using

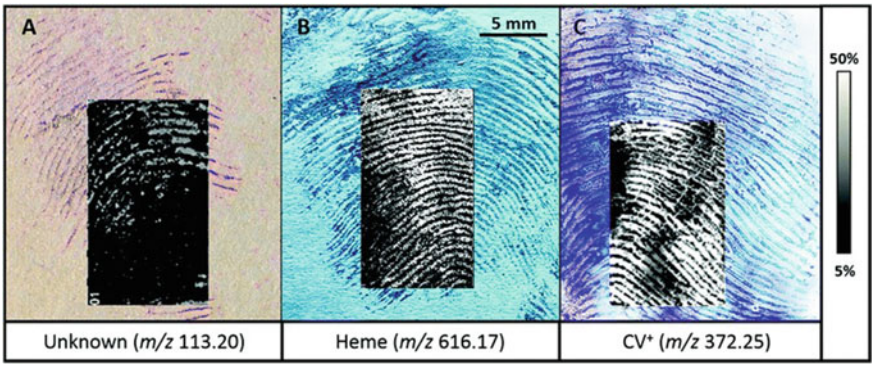

Fig. 1.12 Silver assisted LDI IMS of human bloody fingermarks on nonconductive surfaces after chemical enhancements. Photomicrographs of **a** paper after 1,2-indanedione-zinc treatment, **b** paper after amido black staining and **c** forensic lifting tape after leuco crystal violet treatment. The insets show the LDI IMS of bloody fingermarks acquired after chemical development: **a** unknown compound (*m/z* 113.16), **b** heme (*m/z* 616.17) and **c** CV + (*m/z* 372.25). Republished with permission of the Royal Society of Chemistry, from Lauzon and Chaurand [69]; permission conveyed through Copyright Clearance Center, Inc

the same spraying devices. The authors also highlighted the need to carefully tune the sample preparation to avoid background interferences for the ions of interest.

One of the limiting factors of any mass spectrometry imaging technique is the absence of a chromatography step, which limits selectivity. For liquid samples, conventional approaches use the characteristic retention time alongside mass spectral information for compound identification. In 2015, the Francese's group offered a solution to problem of poor selectivity using imaging mass spectrometry [51]. Here they demonstrated that MALDI coupled to ion mobility mass spectrometry for the detection of cocaine and its metabolite benzoylecgonine in a single fingerprint from a drug user. Ion mobility allows separation of ions prior to mass spectrometry, with each ion having a characteristic "drift time" due to different collisional sections despite the instances in which these ions are isobaric. This gives a more selective method for the qualitative determination of drugs of abuse from a fingerprint. Additionally Rowell et al. [73] and then Bailey et al. [74] showed the possibility to image metabolites of drugs of abuse in a fingerprint collected from drug users (therefore also from non-spiked fingerprints) using MALDI. Future work would of course needs to explore the significance of detecting an illicit substance in a fingerprint using this technique by surveying a greater number of fingerprints from users and non-users.

It has also been demonstrated that MALDI can be used to detect a wide array of illicit drugs and drug metabolite standards in the presence of a fingermark. Groeneveld et al. [66] found that cyanoacrylate fuming and vacuum metal deposition did not interfere with the detection of relevant ions from illicit substances. More recently, Skriba et al. [75] showed that nanoparticle deposition could be used to enhance sensitivity to drugs of abuse in fingerprints. Additionally, Lauzon et al. [69] showed

the possibility of detecting illicit drugs from contact using silver-assisted LDI and compatibility with fingerprint enhancement techniques.

In a recent *Analyst* publication, Bradshaw et al. [57] discuss the complexities, limitations and successes of deploying MALDI in operational situations, illustrating four casework examples of MALDI imaging applied to fingermarks. Whilst not all fingermarks could be imaged (for example when they were especially weak or smudged) the technique was capable of detecting cocaine, cocaethylene and relevant metabolites in a fingermark that had been recovered from the interior of a window frame by using a typical CSI enhancement technique (Fig. 1.13). After an initial denial, the suspect eventually confirmed drinking alcohol and taking cocaine. As yet, there appears to be no information on how likely it would be to find (for example) cocaethylene in the fingerprint of a drug user, or even in a non-drug user and what it says about the circumstances of deposition. Clearly, considerable work is needed in the future to establish a robust framework for interpreting this new type of evidence, though this article does show the ability of MALDI to operate under full operational conditions.

Attempts to determine the age of a fingerprint using MALDI (or indeed any mass spectrometry technique) have had mixed results so far. Francese et al. investigated ageing of artificial fingerprints generated using a silicon fingertip, and a sebaceous reference lipid pad [76, 77]. For these model fingerprints, by carrying out principal component analysis of the entire lipid dataset, the authors were able to show a clear time dependency of the artificial marks, but considerably more work would be needed to apply this to natural fingerprints. Following on from this O' Neil et al. [78] chose instead to capitalize on the spatial information available using MALDI. This group found that using MALDI to image the diffusion of fatty acids and TAGs across fingerprint ridges had a high dependence on the substrate, and this confounds the complexity of fingerprint age determination.

Similarly, Ferguson et al. used detection of peptides and proteins from fingermarks by MALDI, along with the multivariate modelling of the spectra to determine the sex of a donor (based on 80 donors) [79]. This may be useful in the case where a fingerprint is smudged or a donor is not listed on a fingerprint database. The methodology developed by Ferguson et al. enabled the determination of the sex of a donor with 85% accuracy when fingerprints were deposited on aluminium slides. It is yet to be determined whether this methodology is compatible with development processes, but shows another interesting possibility for the future direction of fingerprint analysis.

1.4.3 Desorption Electrospray Ionisation (DESI)

Desorption Electrospray Ionisation (DESI) was conceived in the early 2000s by Purdue University and has been rapidly commercialized and adopted across various sectors of industry outside of forensics due to its ease of implementation and use [43]. The technique can be carried out in ambient air pressures without the need for a space-confining chamber (unlike most SIMS and MALDI systems) and does not

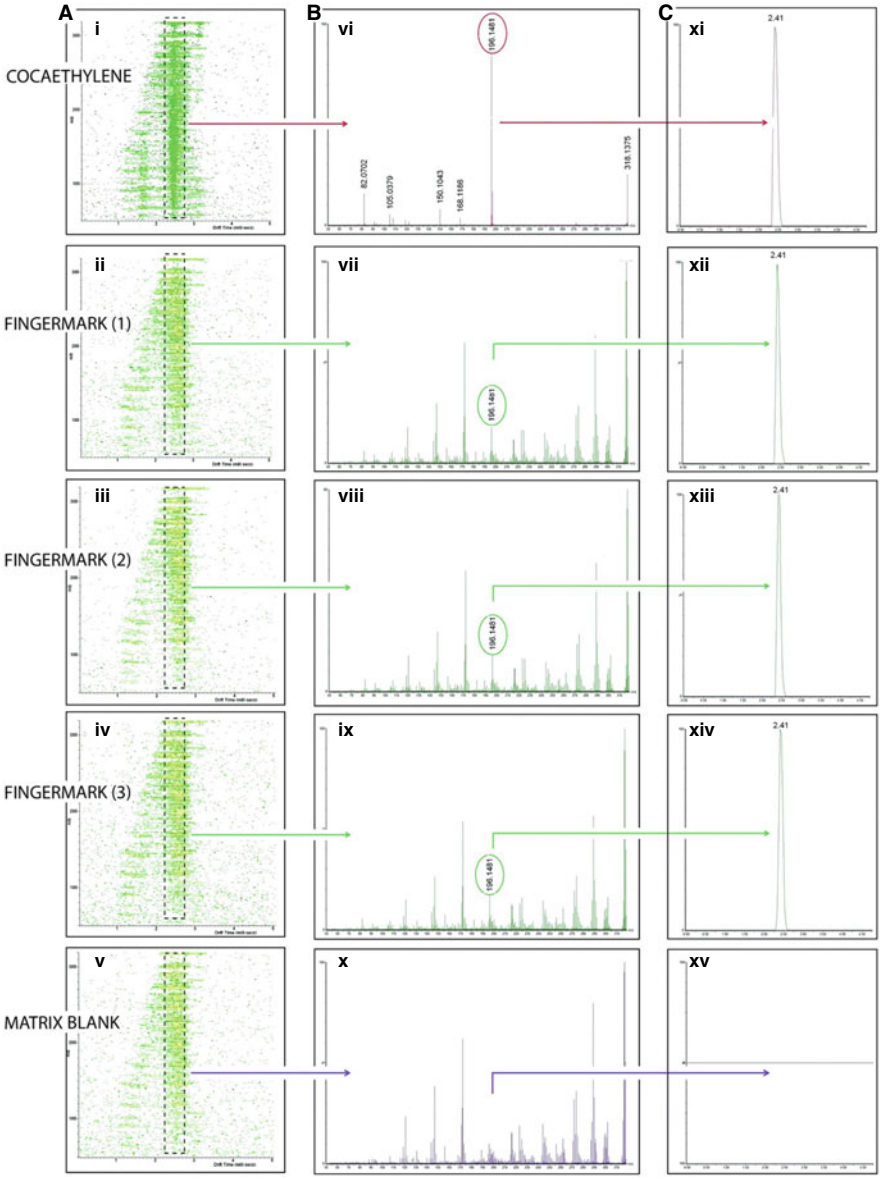

Fig. 1.13 MALDI-IMS-MS/MS analysis of the ion at *m/z* 318.1158 suspected to be cocaethylene (COCE) within a primary fingermark lift recovered on the interior of a window frame following enhancement with carbon black powder. Panel A shows the drift scope plots of COCE standard (i), COCE in 3 location of the marks (ii–iv) and 1 location outside the mark [matrix, (v)] that were generated through transfer fragmentation experiments. Panel B shows the MS/MS spectra of the ion at *m/z* in the COCE standard and in the 4 locations (3 in the mark and one outside it) (vi–x) after the selection of the parent ion and products ion within the same drift time plume in the fingermark and in the standard. Selection of the ion product at *m/z* 196.1481 in common to the COCE standard and COCE in fingermark and matrix shows superimposable drift time chromatograms (panel C xi–xv). Reproduced from Bradshaw et al. [57] Published by The Royal Society of Chemistry

require sample preparation. Therefore in theory, DESI provides a high throughput alternative to SIMS and MALDI, although with a trade-off in sensitivity to large molecules (compared with MALDI) and spatial resolution (compared with SIMS). In DESI, a solvent is pushed through a capillary under a high voltage to make a focussed electrospray. By careful control of the solvent composition, flow rate and geometry, it is possible to obtain a spot size of 20 μm, although more typically image resolutions of 50–100 μm are achieved. The solvent is used to dissolve, ionise and desorb sample analytes from the surface, which are then are swept into a mass spectrometer. The sample is moved under the electrospray to build an image of its chemical composition.

In 2008, Ifa showed the distribution of cocaine on the fingerprint ridges of a donor after contact with cocaine, using DESI [80] and this was the first published example of a mass spectrometry imaging technique yielding molecular images of fingerprint ridge detail. The same group demonstrated the capability to detect explosive residues spiked into a fingerprint [81]. The cocaine image produced using DESI was matched to ridge details on an inked fingerprint. In 2015, Bailey et al. showed that cocaine and its two metabolites, benzoylecgonine and methylecgonine, could be detected in the fingerprints of drug users ($n = 4$) using DESI, using a 2 min spot analysis method [51].

An Application Note from Waters has recently focussed on the use of DESI for fingerprint imaging. The fingerprints of 4 donors were imaged and mass spectral analysis revealed peaks corresponding to monoglycerides, triglycerides, diglycerides and phospholipids [82]. Principal component analysis was applied to spectra extracted from the fingerprint images and showed an ability to distinguish male and female fingerprints. It should be noted here that only two donors of each sex were used to build this model, and that fingerprints were deposited on glass slides without any visualisation reagent. Nonetheless, the work offers up a second possible demonstration that establishing the sex of the donor may be eventually possible.

Unlike MALDI or SIMS, the efficacy of DESI to image developed fingerprints has not yet been tested. The versatility of the technique to image endogenous or exogenous compounds on different surfaces is also unknown, although artificial fingerprints have been successfully imaged on tape lifts containing explosives [83, 84].

1.4.4 Surface Extraction Techniques

Surface extraction techniques offer some of the benefits of both imaging and conventional chromatography analysis. In a surface extraction technique, a probe (for example a pipette tip or nano capillary containing analyte) is used to push solvent onto the surface of the sample. The solvent and dissolved analytes are then re-aspirated into the capillary tip. Techniques for surface extraction include direct analyte probed nano extraction (DAPNe) and Liquid Extraction Surface Analysis (LESA), which has recently been commercialised. The sampling diameter of DAPNe can be as low as a

few microns, whereas LESA operates at the few hundred micron scale. Either method conveys the opportunity to extract analytes from a small area of the fingerprint, thus preserving most of the evidence.

Clemons et al. demonstrated feasibility of detecting fingerprints contaminated with caffeine, cocaine, crystal methamphetamine, and ecstasy [85] as well as RDX and TNT [86] using DAPNe. Bailey et al. showed that heroin, cocaine and the respective metabolites could be detected in the fingerprints of drug users using LESA [74]. Additionally, by comparing data collected using LESA-MS with GC-MS and LC-MS analyses of latent fingerprints, it was observed that a greater number of compounds were detected in a single run. Whilst no work has yet explored the compatibility of the LESA with different substrates or development reagents, LESA may well be suited to exploring chemical changes upon ageing due to the speed at which the analysis can take place (around 5 min per sample) and the high sensitivity to an array of fingerprint constituents.

1.5 Conclusion and Notes on Operational Readiness

The perceived operational readiness level of the techniques discussed above for various applications concerning fingerprints (i.e. for drug testing, where deposition can be controlled) and fingermarks (for forensics, where the deposition is uncontrolled) are summarized in Table 1.1. The scale of operational readiness is categorized as follows: (1) where a successful demonstration has taken place on a surface chosen primarily for its compatibility with the technique rather than operational relevance or demonstrated on a selected (small number) of samples only; (2) where the technique has additionally been tested under operationally relevant conditions (for example after development or on a range of relevant surfaces); (3) where the technique has been additionally applied to a large number of samples; (4) where pseudo operational trials (blind studies, validation exercises) have taken place; (5) where the technique has already been deployed operationally, either in drug testing or forensics. It should be made clear that this table represents only the progress that has been made towards operational deployment, and therefore a technique with a low score is not necessarily unsuitable for a specific application; it may simply have never been tested.

It is worth noting here that both SIMS and MALDI have been included in the recently launched in the 2014 Fingermark Visualization Manual edited by the Home Office [3]. In particular, these techniques were classed in Category C, Technology Readiness Level 3, meaning that the techniques have shown potential to be included within the operational fingerprinting processes, although their full capabilities have yet to be demonstrated.

In toxicology, LC-MS has reached sufficient maturity to be deployed operationally for drug testing, via the company Intelligent Fingerprinting Limited. Similarly, for detecting drug residues and metabolites in fingerprints, MALDI has reached the highest level of maturity in terms of operational deployment, having undergone method

optimisation, validation and finally testing on samples from casework. These techniques are therefore graded a 5 in terms of operational readiness for these applications.

For determining the deposition order of fingerprints and laser printed inks, ToF-SIMS has undergone a laboratory inter-comparison and blind testing exercise. Its compatibility with ninhydrin and indandione developed fingerprints, aged fingerprints and various depletions has also been demonstrated. Additionally, ToF SIMS has been tested for the enhancement of pre-developed fingerprints across a range of substrates, after a range of environmental ageing conditions and after development with various reagents, although it has not yet been deployed operationally. ToF-SIMS has therefore been graded a 4 in terms of operational readiness for both overlapping fingerprints and inks as well as enhanced imaging of fingerprints.

For other applications—namely fingerprint ageing, resolving overlapping fingermarks, sex determination, explosives detection from a fingermark, various mass spectrometry techniques have shown promise, as indicated in Table 1.1. However, the operational readiness of these methodologies is comparatively low, where in general the observations have only been demonstrated on pre-selected substrates or without prior development of the fingerprint. More work is needed to enhance the technology readiness of these techniques.

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Chapter 2
End User Commentary on Mass Spectrometry Methods for the Recovery of Forensic Intelligence from Fingermarks

Stephen Mark Bleay

The application of advanced analytical techniques to the examination of fingermarks has generated much recent media interest because of the wealth of information that can potentially be obtained from such forensic traces. As one of the few types of trace evidence that can be used to identify an individual, fingermarks remain an important tool in the investigation of crime, and still account for appreciably more criminal identifications worldwide than DNA. Because fingermarks have been used for more than 100 years, it may be thought that there are few advances that can be made in the field. However, the potential to go beyond the use of fingermarks for identification and to add contextual information to an investigation is why advanced analytical techniques have become of such interest.

The combination of features that makes techniques such as MALDI, SIMS and ATR-FTIR of such interest for fingermark analysis is the ability to obtain chemical information from the fingermark, and the ability to map the distribution of the constituents at a resolution sufficient to distinguish fingermark ridge detail.

Even if the actual classification of the chemicals present in the fingermark is not of interest, the ability to map the distribution of unknown chemicals that are abundant in the fingermark may be capable of providing additional detections for the criminal justice system. This is because fingermark visualisation processes are optimised to target constituents expected to be abundant in fingermarks, such as the amino acids in eccrine sweat or fatty acids in sebaceous sweat. If the fingermark is rich in another unrelated contaminant that has been picked up on the fingertip, it may not be particularly well developed by a conventional reagent. However, the use of a chemical mapping technique may 'fill in' missing ridge detail and turn a fragment of a fingermark into a criminal identification.

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Similarly, the ability to produce chemical maps may be useful in the long-standing issue of separating overlapping fingermarks. If such marks have been deposited by different people, and each contains different chemicals, it may be possible to produce chemical maps that are unique to each fingerprint donor and allow identifiable fingermarks to be seen in isolation.

Both the above examples describe circumstances where new analytical techniques provide added opportunity to use fingermarks in their traditional application of identification. However, there is a greater potential to utilise such techniques in provision of contextual information that may be relevant to a case. Although this has, to date, been little exploited operationally, the growing range of information that can be obtained and the growing awareness of this capability may mean that this will change in future.

It has been shown that the advanced mass spectrometry techniques now being researched can provide a wealth of information about the lifestyle of the depositor of the fingermark. Even if a contact mark does not contain sufficient ridge detail to identify an individual, it may be possible to obtain supporting contextual information from the chemicals present such as gender, diet and medication that may enable the narrowing down of a field of suspects.

Of equal importance is the ability to identify exogeneous substances picked up on the fingers during handling. If a particular substance is relevant to the investigation, the presence of it in a fingermark could be used to link a suspect to a specific location or source material. The mapping capability of the technique can also add context to the distribution of the substance on the finger, possibly enabling the investigator to distinguish between deliberate and accidental contact.

Other examples of where advanced analytical processes have been used to provide contextual information are “depth profiling”, where it may be possible to determine whether fingermarks have been deposited on documents before writing or printing, or vice versa. This can be an important issue where suspects offer the defence that documents that are currently of an incriminating nature were blank when handled by them.

Although many of these applications have been demonstrated ‘in principle’ in feasibility studies, there are still barriers that need to be overcome before such techniques find wider use on casework. An important aspect that needs to be addressed is that of validation. Forensic science laboratories worldwide are increasingly adopting the ISO 17025 standard, which requires techniques used within the laboratory to be scientifically validated. For a technique to be used in the Criminal Justice System, several aspects of its performance will need to be addressed. The scientific principles that underpin the method should be well understood and published. Where processes are being used for the detection of trace quantities of substances, the sensitivity and selectivity of the process to its target species should be tested, and any interfering substances that could produce ‘false positives’ or ‘false negatives’ identified.

For the advanced analytical techniques now being reported there is already an understanding of the scientific principles, and knowledge of sensitivity and selectivity to fingermark constituents and common contaminants is being developed. What is still required for the processes to be considered sufficiently validated for use in a court room are some larger scale studies, including elements such as repeat experiments,

blind testing and the determination of potential effects on other types of forensic evidence.

Another aspect of forensic evidence recovery, where advanced analytical techniques could provide future benefits, is in the current drive to 'do more at the crime scene'. Mass spectrometry-based methods could find an important application in providing a confirmatory test for substances of interest at the crime scene, potentially saving both time and money by negating the need to send samples back to a laboratory for analysis. However, this would require a validated database of target substances to be generated and increases the importance of conducting an assessment of the impact of potential interferents. The impact of contamination within the instrument will also need to be considered, together with the potential for cross-contamination between samples. Research will need to establish whether contamination can be easily removed when present, and whether it interferes with normal operation for forensic applications.

If crime scene use is to become a reality, thought will need to be given to miniaturisation of instrumentation and its portability. Alternatively, protocols for taking evidence back from the crime scene to laboratory for fixed analysis need to be put in place. The use of lifting media (gelatin lifts and adhesive tapes) has already been demonstrated for removing fingermarks from surfaces for analysis by ATR-FTIR and MALDI. However, it may be preferable to directly analyse marks in situ on the surface of small portable items, which may limit practical application to processes that can operate at ambient pressure and can accommodate reasonably large samples.

Regardless of all the potential operational advantages offered by advanced analytical methods, a question that will always be raised is the cost. It is evident that the cost of such processes will be far higher than the conventional fingermark visualisation and imaging processes used in forensic laboratories, so such methods are unlikely to be used for investigation of volume crime. However, there are circumstances where specific contextual questions need to be answered (e.g. is this mark really in blood? Does the contaminant in the fingermark match a specific substance associated with the crime scene?) and mass spectrometry may provide the answers. For high profile or high priority cases, the additional initial costs of using advanced analytical methods may be justified by providing information vital to a detection, and potentially reducing long terms costs by saved time in criminal investigations.

Chapter 3
Novel Technological Applications for Latent and Blood-Stained Fingermark Aging Studies

Josep De Alcaraz-Fossoul and Meez Islam

Abstract At the present time, there are no standard methodologies to reliably determine the age of (latent) fingermarks recovered from crime scenes. Estimating the time of deposition of this type of evidence is a complex challenge that remains scientifically unsolved in the forensic domain. This chapter addresses the effort to investigate and evaluate the age of fingermarks, and answer the question: how much information can "imaging technologies" provide on fingermark aging? The objective is to introduce the reader to novel applications of existing technologies—Optical Profilometry (OP) and visible wavelength Hyperspectral Imaging (HSI)—that can visualize and record variations in the topography of ridges and follow spectral changes in blood-stained fingermarks, respectively. OP has been typically used for the 3D analysis of surface roughness of materials; whereas HSI has been previously used to detect and identify blood stains in a forensic context and estimate their age in laboratory settings. These non-destructive, contactless, imaging technologies eliminate the need for manipulating friction ridge skin impressions and minimizing sample destruction. Most importantly, they allow the simultaneous collection of qualitative and quantitative data that can be analyzed using spatio-temporal statistical models to investigate the mechanisms involved in ridge degradation. OP and HSI, among other technologies, are establishing new foundational research to integrate the *age* variable in future fingermark examination flowcharts. This inclusion could potentially reduce identification errors that are caused by time inconsistencies between the evidence discovered and the crime committed, as well as maximize the use of resources by decreasing the number of traces to be processed.

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3.1 Introduction

For decades, forensic sciences have adapted scientific principles and technologies from other disciplines for the analysis of crime scene evidence and the identification of suspects. The goal has been to obtain valid proof with maximum objectivity and reliability to associate the true perpetrators with the crime, beyond reasonable doubt during the judicial process. To date, fingermarks remain, together with DNA analysis, the core tool for human identification and are often the fastest and most accessible type of forensic evidence available for this purpose. In addition, fingermarks are more than just a biometric tool; they can also provide chemical trace information about the donor; for example, on the use of illicit or prescription drugs, donor's diet, and health conditions, among others [1–3].

Current friction ridge skin methodologies—mostly fingermarks[1]—identify suspects by their morphological patterns; however, the time of fingermark deposition cannot be reliably estimated and remains an unsolved issue in forensic science [4, 5]. The absence of (international) standards to detect inconsistencies between time of deposition and the perpetration of a crime has a negative impact on the criminal justice system. For example, no information on the age of a trace can invalidate otherwise strong items of evidence or wrongly implicate innocent individuals not directly related to the crime. As a result, many suspects are released and innocent citizens wrongly implicated when fingermark evidence cannot be substantiated in court by the time of deposition (i.e. the age of the fingermark).

The ideal scenario is that in which any investigator/scientist would be able to objectively qualify and quantify changes in ridge topography of unprocessed fresh and aged latent fingermarks and blood-stained fingermarks, either at the scene or in the laboratory. Variations in ridge characteristics over time would be statistically tested and include a measurement of error. Previous studies [6–19] have correlated the time of deposition with certain visual and chemical changes in ridge patterns and the effect of the surrounding environment but with limited and partial results (see Sect. 3.4 for further details). These include research on the chemical composition of sweat secretions [6–8, 12], visual examinations of topographical changes over time [10, 15–20] or chemical imaging of fingermark components [11, 13, 14, 21, 22]. All these studies have provided relevant insights for a better understanding of fingermark aging, but results have shown some limitations. In this chapter, the focus will be on the novel use of Optical Profilometry (OP) to visually characterize and measure latent fingermark ridges in 3D and the potential of HyperSpectral Imaging (HSI) to detect spectral changes in blood-stained fingermarks over time.

Ideally, crime scene investigators would have available easy-to-use, inexpensive, portable and quick analytical tools to estimate the age of latent and blood-stained fingermarks. To this day, and from a scientific perspective, the development and

[1]This term refers to latent or invisible impressions (undetectable to the naked eye), plastic or molded impressions (e.g. prints on clay or wax) and patent (aka visible prints) unintentionally made by friction ridge skin on a surface. They require a specific enhancement or processing for analysis. The term *fingerprint* refers to controlled setting (e.g. inked prints taken from detainees).

operational deployment of these very desirable technologies have been difficult due to several factors which include:

- *Environmental challenges*—Numerous environmental variables may affect fingermark degradation and these pose a complex challenge to determining which one(s) is/are most significant in the aging process. Extrapolation of data in the form of a significant population study has also proven to be an experiment design limitation.
- *Limited financial resources*—Trends in innovation have traditionally focused on other branches of science, such as genetics, while forensic science has fallen behind, in part due to limited and dedicated funding.
- *Technological barriers*—Recent technological advances have enabled the development of economically affordable, easy-to-use software and hardware for many mobile applications. Despite these advancements, no commercial computer-based tools are yet available for dating latent fingermarks due to the aforementioned issues.

From the range of currently available technologies to be used for latent fingermark aging studies, an Optical Profilometer (OP) was chosen for its potential and already proven capabilities in analyzing very small surfaces in 3D. OPs have been used for years in the visual study and data collection of surface roughness of materials, proving to have many advantages and a few limitations [23]. Optical Profilometry is a non-destructive, contactless, three-dimensional (3D) microscopic imaging technology that is presented in this chapter as a novel tool for analyzing latent fingermark ridge topography [23]. One of the key features of an OP is that it eliminates the need for enhancing (e.g. by powdering) latent fingermarks to visualize them in a research context; minimizing specimen manipulation and avoiding the introduction of potential error in ridge measurements. It also allows the simultaneous analysis of several visual ridge features (e.g. ridge height, area, volume and width) beyond the simple visualization of ridges. Figure 3.1 shows a typical equipment setup.

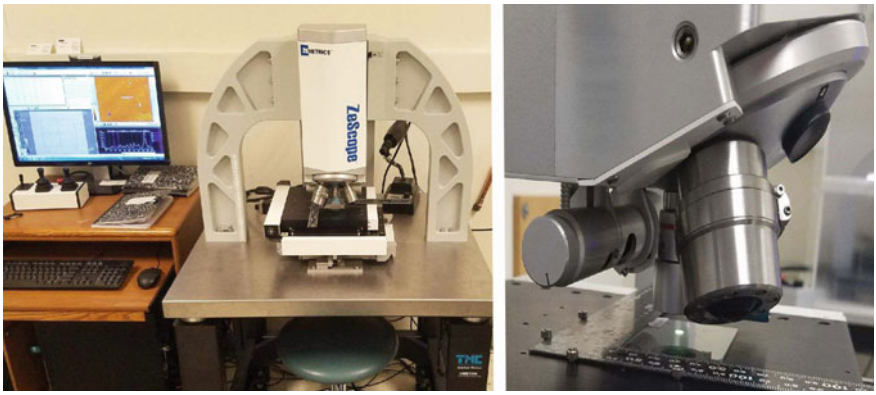


Fig. 3.1 Typical optical profilometer set up (left) and detail of microscope objective (right) with a microscope glass slide mounted on the moveable platform

The other technology covered in this chapter is hyperspectral imaging (HSI) and will concern blood-stained fingermarks. This technique can only be applied when blood is present. In a forensic context, blood is found to be the most common contaminant of fingermarks. An assumption of the technique is that the age of the fingermark is the same as the age of the blood stain. However, the technique cannot determine whether the fingermark was made in blood or was deposited sometime before and, then, subsequently covered with blood at the time of the crime. HSI is an established non-contact, non-destructive technique which was originally developed for satellite-based monitoring of the earth but has recently been used in forensic applications, including the detection, identification and age estimation of blood stains [24, 25]. It is based on recording images at series of wavelengths and combining the information to create a 'datacube' such that spatial information is recorded in x and y dimensions and spectral information is recorded in the z direction. Thus, in a hyperspectral image, every pixel in the image also contains spectral information across a range of wavelength bands. This information can be used to detect and identify substances in an image by statistically comparing the spectrum in the pixels against a database reference spectrum. HSI can be performed in several ways and also across different wavelength ranges from the ultraviolet (UV) to the infrared (IR). The most commonly used and most accessible wavelength range is the visible range from 400 to 700 nm while one of the simplest ways of obtaining visible wavelength hyperspectral images is to attach a liquid crystal tunable filter (LCTF) in front of a monochrome camera and scan across the wavelength range while recording images at each wavelength. Custom software can then be used to create and analyze the resulting 'datacube'. Figure 3.2 shows a typical equipment setup.

Constant technological advances, our better understanding of latent fingermark chemistry, and of the effect of the environment on topographical changes of ridges over time, make age estimation a more feasible endeavor. In order to accomplish this objective, it is of paramount importance that scientists from diverse disciplines (e.g. biology, chemistry, forensic sciences, statistics, computer science, etc.) come together in multidisciplinary collaborations to share and build knowledge from their respective fields.

A robust estimate of a fingermark's age could be used in the near future as admissible data in court, increasing the probative value of evidence, with a direct positive impact on public confidence in criminal justice. It would also fulfill an emerging demand for obtaining rapid analytical results as well as tackling current scientific, legal and societal demands that have weakened the reliability of forensic evidence in the judicial system. The technologies presented in this chapter demonstrate the significant advances in forensic research in the field of fingermark aging that could be used towards increasing robustness of this type of evidence.

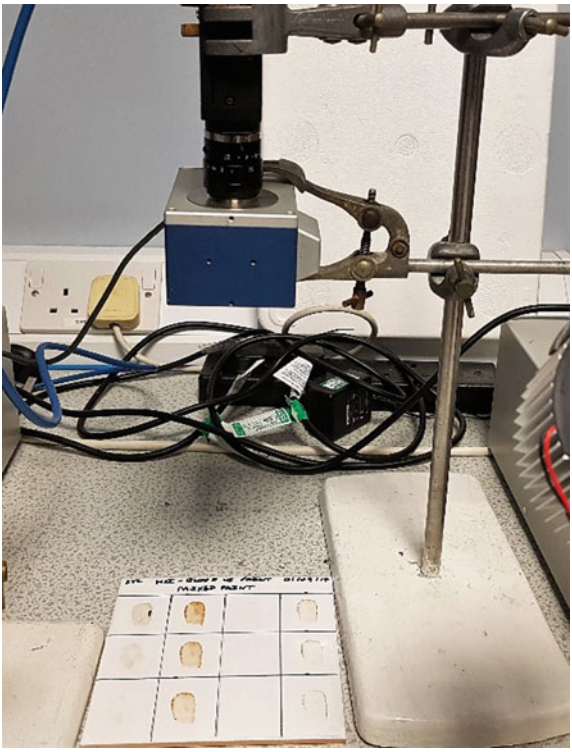


Fig. 3.2 A typical HSI setup based on a LCTF attached to a camera

3.2 The Impact of Fingermark Identification Errors

Fingermarks have aided in the resolution of millions of criminal cases worldwide for over a century. However, in our modern technological society, errors in identifications have become a grave concern with many being caused, in part, by the examiner's subjective analysis and/or conclusions during the process of "matching" the unknown fingermark to its donor [25–28]. We can ascertain with high degree of confidence that these errors are not caused by the intrinsic identification power of the trace itself. The degree of subjectivity on the analysis and the reported errors in biometric identifications have caused much controversy in the criminal justice system and beyond. It was, in fact, brought to the forensic science community attention by the National Research Council (NRC) report in 2009 in the US,[2] among other reports published around the same time, or soon after, in other countries.[3] These

[2]Strengthening Forensic Science in the United States: A Path Forward. Committee on Identifying the Needs of the Forensic Sciences Community, National Research Council. 2009. ISBN 978-0-309-13135-3.

[3]Issues also noted by the Scottish Report "The Fingerprint Inquiry"; APS Group Scotland. 2011. ISBN: 978-0-85759-002-2.

reports strongly criticized the absence of reliable scientific methods in some forensic disciplines that led to mis-attributions and mis-identifications of fingermarks.

In the context of this chapter, mis-attribution and mis-identification refer to different closely related concepts (Fig. 3.3). The former denotes cases where a fingermark is correctly associated to a suspect but is inconsistent with the time the crime was committed, while the latter matches incorrectly the trace to a donor because not enough clear identifiers are present, i.e. poor quality and/or quantity of necessary ridge detail; in this case, an error in interpretation by the expert has been made, leading to a wrongful match. Despite standardization of the fingermark examination process (ACE-V[4]) and the development of protocols in crime laboratories, these types of errors still occur.

Regarding mis-identifications, false positive rates[5] are estimated at 0.1–3% depending on the sources, mostly from proficiency tests [28–30]. For example, according to data published by Simon A. Cole [28] based on Federal Bureau of Investigations (FBI) research in the US, an estimated 0.8% false positive rate exists in the current methodology for analysis, i.e. the incorrect association of a person with a recovered fingermark.

Whilst mis-identification remains an issue, the most common situation arises when a suspect has legitimate access to a scene before or after the actual crime; then, the inability to date the time of fingermark deposition often invalidates otherwise strong pieces of evidence or wrongly implicates innocent individuals. Issues of reliability of fingermarks as a means of human identification frequently arise in court when evidence cannot be directly correlated to the moment a crime was committed [5].

But, how much do these identification errors impact our society? For example, a Eurostat report by the European Commission[6] indicated that there were over 28 million serious crimes reported in the European Union in 2008, impacting, directly or indirectly, over 75 million individuals.[7] Of these crimes, and based on typical figures,[8] it could be estimated that fingermarks were recovered from approximately 30% of cases, and of these, and in the absence of official global figures, a conservative average of at least 25% would be positively correlated to a donor. In Europe each year, 2.1 million offenders are identified by fingerprinting, and although there are no official reports on error rates, it could be estimated that nearly 17,000 cases annually may result in false positives due to both mis-attributions and mis-identifications. Different areas of the criminal justice system could benefit from age evaluations of

[4] ACE-V: Analysis, Comparison, Evaluation, and Verification, referred as being a standard scientific method in the comparison and identification of friction ridge impressions.

[5] Refers to the prevalence of cases that incorrectly associates a person to a recovered fingermark.

[6] http://ec.europa.eu/eurostat/statistics-explained/index.php?title=Archive:Crime_and_criminal_justice_statistics,_data_2008-2013.

[7] As mentioned on page 141 of the REGULATION (EU) _o …/2013 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of the Horizon 2020—the Framework Programme for Research and Innovation (2014–2020).

[8] ANZPAA NIFS (Australian New Zealand Policing Advisory Agency) report "End-to-end forensic identification process project", in 2012.

Fig. 3.3 Diagram depicting types of error and how the inclusion of the "time" factor can improve the reliability of evidence

fingermarks; for example, by decreasing the number of traces to be processed, expediting the prosecution process and minimizing exposure to primary and secondary victimization[9] of individuals directly or indirectly related to the crime.

It is clear that fingermark examinations have come with errors as well as bias [31] which must be addressed. However, there are some valuable aspects of using the cognitive system in the process of fingermark analysis. As Dror mentioned in 2013 "*expert opinions can be valuable in court, not for being fact but simply as a matter of only [expert] opinion*". Another positive aspect is that a degree of subjectivity and cognitive nature can increase the value of these decisions by introducing experience and instincts into the already standardized and successful processes. This involves taking counter measures that can help minimize the contextual influences, biases and even the possibility of error [32]. There is no harm in being a human (subjective) science, the cognitive underpinning of decisions just needs to be understood by the investigators when making an arrest, by the forensic scientist when preparing a report as well as by the jury in court. As individuals become experts, it can be helpful to determine how shortcuts are taken, past experiences are more relied on and how certain marks are examined more selectively due to their initial appearance to the examiners. Once the cognitive architecture of human experts in fingermark forensic science is understood, there can be steps taken in developing best practices [31], as well as implementing a statistical, quantitative aspect to the science. Given the widespread use of fingermark evidence and the public demand for higher scientific standards, it is critical that the methodology for analysis becomes as accurate and error free as possible. Fingermark aging can provide a degree of knowledge and intelligence to help reduce error in identifications.

There has been no shortage of critiques that have cast doubt on the practice of fingermark identification and their use in court. There have been strides made since the publication of the National Research Council Report in 2009, and with these advancements have come more areas of expertise that can yield great knowledge. Fingermark research has developed to encompass incorporating the following:

- Various statistical models for different aspects of fingerprinting;
- Qualitative and quantitative data to support fingermarks as evidence;
- Methods to estimate the age of a fingermark.

Convictions can hinge on the unknown factor of whether or not a particular mark was left within the timeframe of a crime. This is one of the reasons research has taken form to investigate an accurate model for determining the age of latent fingermarks [34] and place a suspect within the timeframe of a crime beyond any subjective witness or victim statements. With the information that new research is providing, the errors made and controversies that emerged in (latent) fingermark decisions could greatly decrease in the years to come. The evolution of an age-old science has gained

[9]Secondary victimization (also known as post crime victimization or double victimization) is the re-traumatization of the (sexual assault, abuse or rape) crime victim. It is an indirect result of assault which occurs through the responses of individuals and institutions to the victim. The types of secondary victimization include victim blaming, inappropriate behavior or language by medical personnel and by other organizations with access to the victim post assault [33].

great steam as research has taken off in the last decade. There is great hope for what forensic scientists have yet to uncover with the knowledge that has already been obtained.

3.3 The Estimation of *Age* Will Improve the Robustness of Fingermark Evidence

The possibility of estimating the age of fingermarks has been implicitly or explicitly discussed in at least 28 court cases from 1961 to 2011 as reported by Girod et al. [2]. The study reported, that, in some instances, experts stated that it was impossible to determine the exact age of fingermarks. However, they provided the court with personal opinions based on experience. Opinions were (and are) subjective in nature, with potential for biases. Also, the majority of the literature on fingermark analysis has emphatically stated that it is "impossible" to precisely determine the age of (latent) fingermarks [35] and it has been recommended that "*(…) age estimation should never be based solely on the quality of a developed mark (…)*". Despite these objections to fingermark aging, researchers have studied and established evidence of degradation processes, including qualitative and quantitative visual methods, and chemical profiling of fingermark deposition compounds [2–19, 36–38].

We advocate for the inclusion, in the near future, of the factor *age* in the fingermark examination process (Fig. 3.4), because it could significantly improve the overall robustness of evidence beyond the most immediate attribute of estimating a specific time of deposition. The increased value of the probative power of evidence could be summarized as [37]:

- Temporal location of a suspect during the perpetration of a crime: the order in which objects were touched and, therefore, the sequence of certain events could be inferred.
- Screening of relevant or priority fingermarks: age estimation offers the possibility of pre-selecting high-priority processing fingermarks from irrelevant ones (low-priority) to prioritize lines of investigation with respect to relevant time periods. Indiscriminate submission (and storage) of "questioned" fingermarks to databases would become limited to a pre-selection of fingermarks with respect to time periods of interest. Age estimation would therefore diminish the potential for biased results when conducting identifications.
- Age distinction of overlapped fingermarks: If the prints can be adequately separated (as it has been demonstrated by Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) [10]), the proposed age estimation scheme could be applied to each print separately and the age of both could be compared.
- New applications of existing technologies: for example, OP is broadly used in material science to analyze surfaces. New developments in the instrumentation could make equipment affordable and potentially portable to the scene.

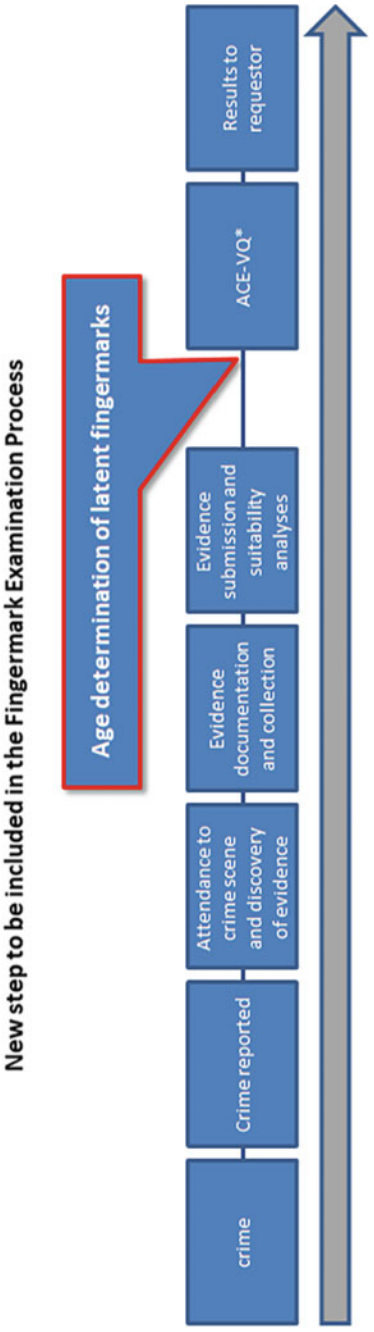


Fig. 3.4 Fingermark examination flowchart. Highlighted is the new stage suggested to be included in the future

From a more general perspective, the criminal justice system and the general public could directly benefit from the inclusion of the *age* factor because it has the potential to:

- Improve the efficiency of public financial resources devoted to criminal investigations by giving crime laboratories a new tool for in situ crime inspections;
- Minimize errors in the identification of suspects by placing them outside the timeframe of a crime;
- Reduce the amount of evidence necessary to secure or overturn a conviction, thus reducing redundant crime evidence analyses and backlogs;
- Speed-up and reduce the cost of judicial processes which involve fingermarks as crime evidence;
- Improve the quality of criminal justice system services by decreasing the annoyance to victims and individuals indirectly involved (secondary victimization) whose testimony is no longer required;
- Reduce overall public administration expenditures.

Moving forward, there are still many skeptics who believe age estimations of (latent) fingermarks is an impossible endeavor. There is still a lack of consensus and a need for more research before a worldwide protocol and process can be standardized for this science; however, in the authors' view, this is not impossible. We agree when Girod et al. (2016) [5] propose that research needs to focus on the chemical, physical and quantitative aspects of fingermark analysis. Researchers need to continue to be open and transparent when it comes to the limitations as well as potential of the (latent) fingermark aging methodologies. The research into fingermark degradation to eventually be able to produce age estimations has come a long way since the National Research Council Report in 2009. Forensic scientists, statisticians, law enforcement officials and other researchers have put great effort into making fingermark analysis more reliable and it has led to the study of fingermark aging processes and the formulation of statistical parameters for reference. Once this discipline is further developed and understood, there is no doubt it could help lessen the time and effort spent on fingermark examinations in laboratories as well as minimize wrongful convictions related to time inconsistencies. This field has grown significantly in the past decade, and it will become more robust and provide a great source of knowledge as time and research continues.

3.4 Technologies and Methodologies Applied to Latent Fingermark Aging Studies: Qualitative and Quantitative Analyses

The natural degradation process of latent fingermarks occurs through intrinsic biological, chemical and physical processes and interactions with extrinsic factors, such as the type of substrate—surface of deposition—and other environmental factors [12,

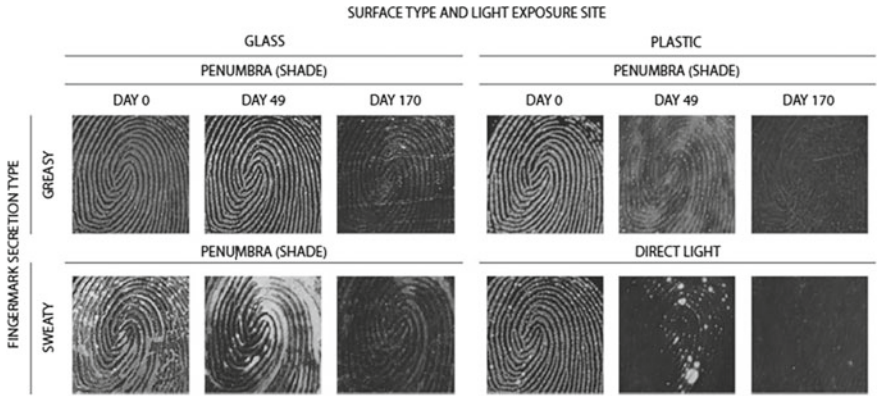


Fig. 3.5 Example of visual latent fingermark degradation patterns dusted with titanium dioxide powder after exposure to different environmental conditions. Figure reprinted with permission from [17]. Copyright 2016, Jon Wiley and Sons

39]. The various research that investigate the aging of fingermarks typically explore chemical and physical (visual) changes. Some of the methods have involved powdering techniques [12, 13, 40], changes in fluorescence wavelengths and intensity [41], changes in chemical profiling [11], as well as electrical methods exploring decay of electrostatic charges [42]. The practical use of these techniques in casework is a concern shared by many scientists in this field. Wei et al. [21] identified the importance in not just studying the visualization and aging of fingermarks but being able to develop reliable methods/technologies that are portable to a crime scene or a mobile laboratory setting [19].

The proof-of-concept of an affordable, rapid, and easy method for estimating time of deposition is technically possible using visual examination alone, as shown in the example depicted in Fig. 3.5. For law enforcement, a visual test for a fingermark's age in situ would prove very useful for crime scene reconstruction. Then, in addition to the *what* and *how* aspects of crime evidence, now the potential ability to include the *when* is not far reaching as a standard in investigations worldwide. Here we also describe examples of selected technologies that have been used to date for age estimations. It is not intended to be a comprehensive review but a mere illustration of the potential of these technologies in obtaining relevant data.

3.4.1 The Application of 2D Imaging to Aging Studies

Research published by De Alcaraz-Fossoul et al. [14], showed the basic concept of characterizing the degradation process of latent fingermarks, under certain environmental conditions, by visual (qualitative) means using a traditional powdering technique (Fig. 3.5) [12]. A quantitative method was developed shortly after that could

statistically model the degradation process [14–17]. Statistical data were obtained from the measurement of four visual parameters observed during the progression of degradation: (1) changes in the width of ridges; (2) decreasing color contrast between ridges and furrows; (3) increasing number of ridge discontinuities; and (4) decreasing number of observable *minutiae*.

One of the most significant findings of this aging experiment was that in some instances, and contrary to common expert belief, there was no significant visible differences between those marks aged and exposed to direct sunlight and those kept in complete darkness. For example, sebaceous-rich depositions on glass appeared astonishingly clear for identification purposes after 6 months (fresh-like appearance); however, the plastic substrate produced marks with more diverse array of results. Other conclusions from this study were in regard to the differences between sebaceous- and eccrine-rich depositions. Understandably, the "greasier" sebaceous specimens were more resilient to degradation after 6-months of aging than their counterpart eccrine-rich depositions. Despite all the relevant discoveries using this 2D methodology, it cannot not provide a complete picture nor could it be used to fully understand and model visual degradation patterns. This is because a fingermark is not a 2D object but a "live" 3D structure deposited on a substrate.

3.4.2 The Complexity of Fingermark Chemical Composition Studies

Fingermark chemical comparisons may become an effective tool to criminal investigations in the future. This showcases the importance of further understanding what a fingermark is composed of and how this may affect the process of degradation and aging. One of the major problems in studying the chemical composition of (latent) fingermarks is the complex task of identifying a stable sweat compound (protein or lipid) that could be used as an aging reference or standard. From the large array of techniques, we will focus on a selected variety. For example, some authors have proposed the use of thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to study the behavior of lipids over time [3, 5], but the results were not conclusive due primarily to technical limitations. Fourier transform infrared spectroscopy (FTIR) has also been used but so far with contradictory results [43].

Gas Chromatography/Mass Spectrometry (GC-MS) is destructive to the prints, but it allows for the analysis of many compounds from different substrates [6, 44]. GC-MS is an analytical method that combines the features of each technique to identify chemical substances within a test sample [45]. The GC-MS method is sensitive to amino acids that are challenging to detect or not detected by other techniques and an extensive range of fatty acids, as well as squalene. Similarly, liquid chromatography/Mass Spectrometry (LC-MS) is a technique used to separate the constituents in a liquid-phase mixture, to identify and quantify components.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is a soft ionization technique. The combination of a laser and a UV absorbing matrix allows the analysis of a variety of molecules. MALDI profiling and imaging for the detection and mapping of lipids, peptides and proteins, and amino acids in fingermarks have been shown [46–50]. This technology has demonstrated to be sensitive for the visualization of groomed, ungroomed and natural fingermarks. Good quality images of fingermark ridges were obtained based on the chemical profile of the deposition. Initial studies reported by Francese's group indicated that this technology could be used for short-term age estimation, (up to 8 days) [51] although the method was only used on "lipid pads" and not real marks. It is believed MALDI has the potential to bring new insights to the field of fingermark aging and is especially useful for chemical imaging of aging lipids [11, 46, 49, 50]. However, this equipment is, as of today, costly and requires high operator expertise.

As the focus of chemical composition is further examined, it could yield important information that could be used to estimate the age of latent fingermarks, such as specific intermediates, concentrations of various fatty acids, etc. If this type of study is conducted on a large scale and even take into account factors such as age, sex, and race, there could be a wealth of information to help further the efforts to estimate age of a latent fingermark. The design of adequate technical protocols is necessary to distinguish between short- and long-term fingermark age estimations because the techniques to be used (visual vs. chemical vs. chemical imaging) have different degrees of sensitivity to degradation changes. For example, MALDI and GC-MS/LC-MS techniques will detect changes that occur after a few minutes/hours to several days of deposition (although currently at an immature stage to scene application), whereas visual changes will assess a timeframe of several days to weeks/months [12]. These techniques provide complementary information of the aging behavior. The combination of short- and long-term aging studies is of interest because fingermarks of very different ages can be potentially found at any crime scenes. Cadd et al. [39] mentioned that distinguishing donor characteristics as well as determining the specific age of a fingermark needs to be distinct priorities to bridge the large gap in forensic science knowledge. It is also important to take into account the substrate, composition and environment when studying (latent) fingermarks because each one of these variables could cause acceleration or deceleration of the degradation process [39].

The current dilemmas in the reliability of identifications have persuaded academics to begin studying the degradation and aging patterns of (latent) fingermarks from different methodological approaches. Having a deeper knowledge in this field can help provide more statistically relevant information to CSI, detectives and prosecutors in their combined efforts to provide time-based evidence beyond a reasonable doubt in court, minimizing wrongful convictions and exonerations.

3.4.3 Approaches to Non-invasive 3D Analysis of Latent Fingermarks

One of the ways research into latent fingermark age estimations can move forward is by shifting from a 2-dimensional train of thought into the 3-dimensional (3D) objects fingermarks truly are. The ability to visualize fingermarks in 3D gives researchers new features and metrics in terms of discovering aging patterns. Non-invasive methods to obtain high definition images have been used in several studies in fingermark aging. Image extraction and comparison methods could clearly benefit from the study of the various ridge changes that occur over time. Liu and other researchers have noted the evolution of fingermark techniques, showing that it is possible to use the 3D aspect of fingermarks to develop a more comprehensive aging model [52].

Merkel and his colleagues were pioneers in examining the topography of an individual latent fingermark by using 3D technology [7]. Merkel understands that there is some aspect lacking in order to provide a definitive age for a fingermark and believes that looking at the print with all three dimensions can help find the solution to this problem. In his research, Merkel does an excellent job at encompassing many different types of conditions in order to account for the variability that is seen throughout the various crime scenes on a daily basis. Looking towards the future, Merkel's new perspective can be used to continue and conceptualize further instrumentation, techniques and methods to help visualize and analyze latent fingermarks in 3D.

Merkel et al. [10] used Chromatic White Light sensors (CWL) and Confocal Laser Scanning Microscopy (CLSM) for image acquisitions. However, these techniques produced low quality images and were not ideal for topographic studies in 3D [12]. Ridge height functions, while derivable, were not reliably or automatically estimated or obtained. CWL and CLSM were also time-consuming imaging techniques, taking about 3–5 h to scan 20 images. Loss of image contrast was another unsolved issue that significantly reduced the quality of the prints. In another study, Dorakumbura et al. [20] used Atomic Force Microscopy (AFM) to observe adhesion and topography in 3D [18]. AFM had an advantage over CWL and CLSM because it rendered better resolution of images at the nanoscale. However, AFM had direct contact between the sample and probe, making it an invasive and potentially destructive method and required perfectly flat surfaces. Additionally, it took AFM longer to scan an image (3–4 h) and it was not used for aging purposes. Stoehr et al. [53] used energy dispersive x-ray spectroscopy, environmental scanning microscopy (ESM) and OP to determine the composition of fingermarks. Again, no aging studies were conducted. X-ray spectroscopy and ESM were also not capable of measuring the height of the sample. Lateral resolution of the ESM was too high for the analysis of ridges at a microscale, while X-ray microscopy required the analysis of large quantities of images. In Stoehr's methodology for OP, fingermarks were sputter-coated or treated before scanning, making it an invasive technique that altered the sample.

3.5 Optical Profilometry: A Tool to Uncover Latent Fingermark Aging Patterns in 3D

As seen earlier, fingermark degradation patterns have been investigated using a multitude of methods that range from studying chemical changes and applying immunolabeling, to visual patterns and effects of photo and thermal degradation. From these options, the constant development of visualization methods continues to provide more knowledge that will help forensic scientists in establishing a reliable standard to estimate the age of fingermarks. Overall, the ability to visualize fingermarks in different ways (e.g. 2D vs 3D imaging) provides complementary information regarding individual characteristics of ridges as they age.

Optical profilometry (OP) is a non-destructive, contactless, 3D microscopic-imaging technology that eliminates the need for developing latent fingermarks (e.g. by powdering) at each data collection time point, thus minimizing sample manipulation and measurement error. OP allows discrete statistical data collection (i.e. as time-lapse) of topographical changes over time. From an experimental approach, OP could be developed to formulate a predictive, statistical model that estimates the age of a fingermark as a function of its topography—height, width, and other morphological parameters—time, and deposition conditions. Spatiotemporal modeling simultaneously considers spatial, temporal, and inter- and intra-subject variability and therefore can produce robust predictive models. OP provides both qualitative (i.e. 2D + 3D images of specific aging stages) and quantitative results (i.e. measurable topographical data). In addition, the analysis of the exact same sample over time allows a time-lapse image capture of the degradation process helping understand the "live" aging process.

The OP uses white light interferometry to determine the height, volume and widths of features on the surface of the sample. A portable version is already in the market and could be potentially used at crime scenes. A CCD—charge-coupled device—camera records the image of the sample as the distance between the sample and the objective is scanned. As the microscope moves vertically, a digital image is recorded. The microscope uses interferometry objectives which split the light into two paths, one path reflected by the sample and the other by a reference mirror. When both path lengths and the same interference fringes appear, a software determines equidistant points between the image and the mirror. A cloud of (x, y, z) vectors is generated to create a 3D digital surface which can be processed using a wide range of programs. The x-y resolution is determined by a chosen magnification. The z resolution is independent of the objective and is extremely high i.e. less than 1 nm. The instrument is equipped with a 200 × 200 mm motorized stage that facilitates large number of samples to be measured sequentially and ensures that the same area is measured at each time point.

This instrument can also reliably provide functions of heights and widths along specified cross-sections in an untreated or raw (latent) fingermark, as shown in Fig. 3.6, with the height function of a cross-section (shown as a line) displayed at the bottom of the image. The heights are measured in micrometers and are relative

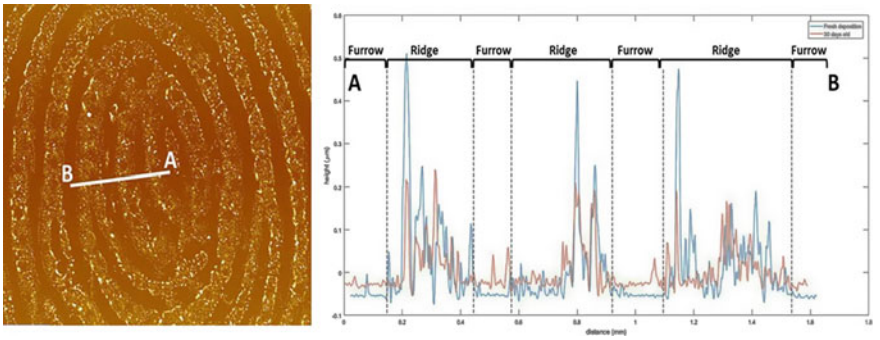


Fig. 3.6 Example 2D/3D image (left) and height functions (right) depicting the differences in ridge heights between fresh (in blue) and aged fingermarks (30 days) (in red). This sample is the middle fingermark of the male donor deposited on glass substrate and aged in darkness. Note that ridge peaks have "eroded" within the 30-day period

to a baseline or an average. Hence, negative values in the height function are expected to be observed. This does not affect data analysis as the height functions themselves will be compared across time in an aging experiment.

OP can also obtain the average height of any given surface area, known as S_a parameter (Figs. 3.7 and 3.8). An image in 3D and topographical data is obtained in 3–4 min on the desired area, typically ~30 mm^2. In aging studies, the same location and area size is used. It must be pointed out that the area is always maximized to cover as much viable fingermark surface as possible within the limitations of the

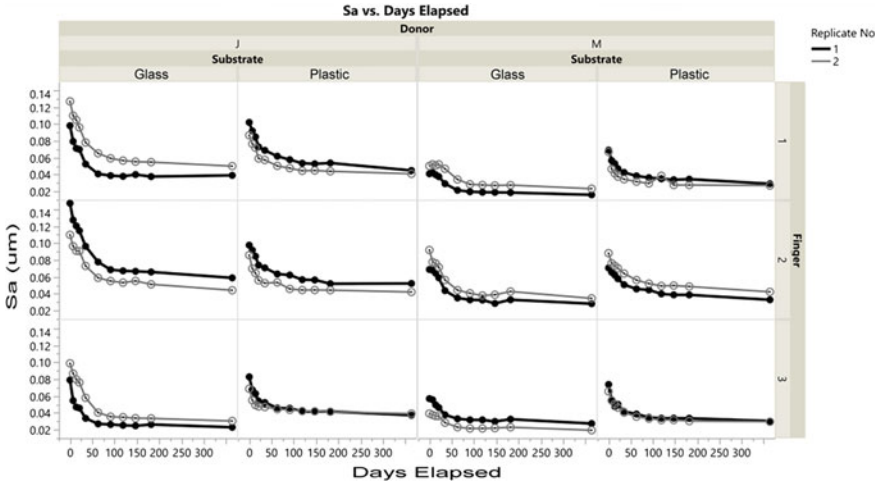


Fig. 3.7 Average height surface (Sa) values (in micrometers) of untreated latent fingermarks (shown as replicates) visualized with OP over time. Figure reprinted with permission from [22]. Copyright 2018, Jon Wiley and Sons

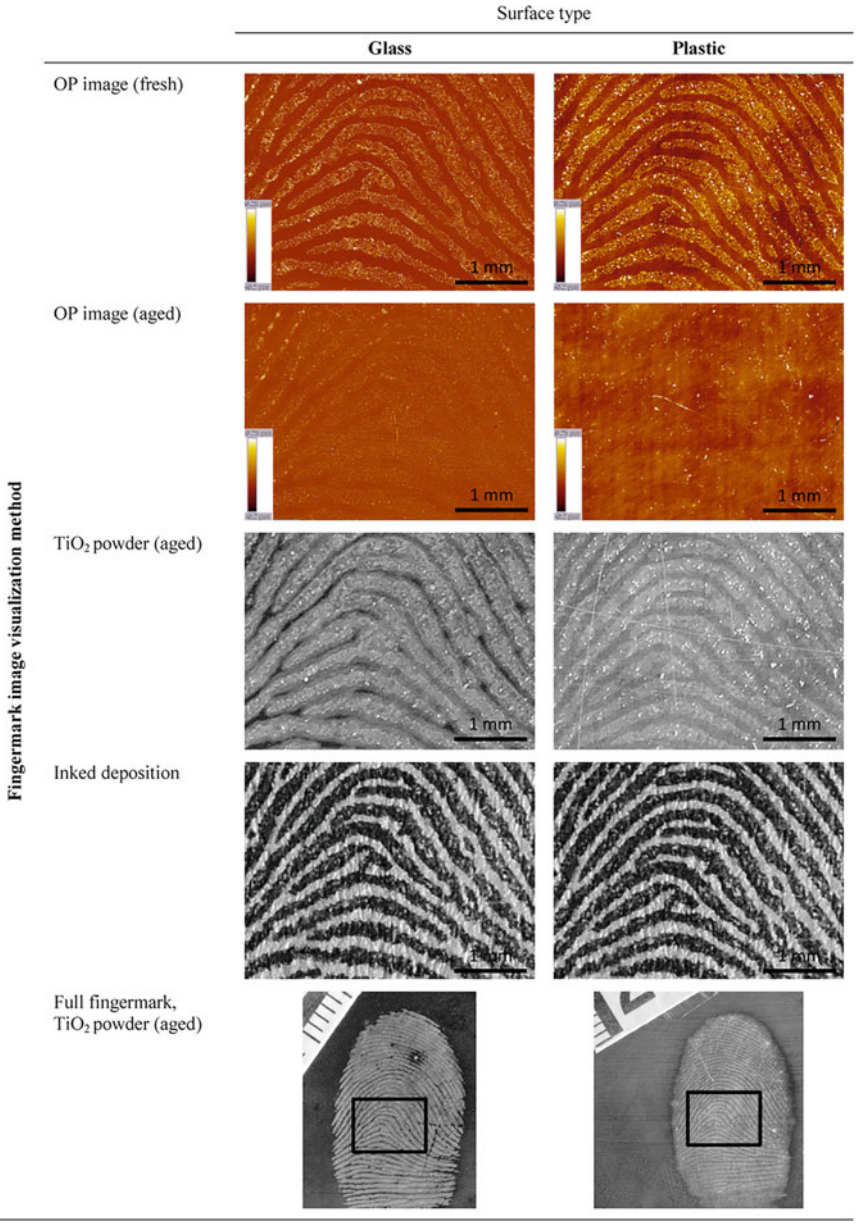


Fig. 3.8 Latent fingermark images from a female donor captured with optical profilometer (OP); the exact same print powdered with titanium dioxide TiO_2 (ridges shown in white); and deposited with ink (ridges shown in black). The aged impressions are visualized one year after deposition. Note the "halos" around the aged latent fingermark ridges in powdered prints. Figure reprinted with permission from [22]. Copyright 2018, Jon Wiley and Sons

microscope (i.e. larger surfaces require longer collection times). In our laboratory, non-porous surfaces, including glass, plastic and metal have shown to be amenable for analysis by OP. The object size requirements are comparable to any standard compound microscope; in our case we used thin surfaces up to 1 cm in height. There are a few disadvantages of the OP, such as the slow data acquisition times if used at high resolutions (i.e. not necessary for our aging purposes); its detection limit (i.e. insensitive to very thin layers of sweat components that can still be detected with powdering); and its high sensitivity to imperfections or "waviness" of the substrate that can mask results. These limitations could be addressed with image processing software, although non-flat surfaces—wavy at a microscopic level—would more than likely prevent (or significantly complicate) any OP analysis no-matter the level of image processing. Despite these restrictions, and compared with other techniques, OP is a sufficiently sensitive fingermark dating technology that provides precise measurements in all dimensions coupled with high quality images.

From an end-user perspective, data acquisition and interpretation require some specific training (few hours) but no prior experience with OP or expertise in microscopy or statistics. High quality images, in 2D and 3D, and data on ridge surface roughness can be collected, transferred and edited using other commonly used software. Depending on the needs of the user, further image and data processing may be desirable which require expertise in 3D imaging and statistical software such as MATLAB®. As OP software evolves, it becomes more user-friendly and versatile in options. Bench top and portable optical profilers are already available in the market from different manufacturers at reasonable prices, making this technology a solid candidate for operational use at crime scenes in the future.

The work described by Merkel et al. [37] and De Alcaraz-Fossoul et al. [22] lead to great hope for age determination of fingermarks, focusing on visual parameters and the three-dimensional ridge features. In future studies, there is the need to include larger populations of donors that can help overcome limitations due to sample size and the unknown effect of variables in order to become statistically relevant. Moving forward, these visual methods must combine the evaluation of biological/chemical, environmental and substrate differences and interactions when it comes to the rate of degradation. Determining the causes of acceleration or deceleration of degradation is critical in predicting aging patterns for latent fingermarks.

3.6 Age Estimation of Blood-Stained Fingermarks

At scenes of violent crime, blood is one of the most commonly encountered types of biological evidence [54] and is the most common fingermark contaminant [55]. The initial objective when dealing with suspected blood evidence is to conclusively establish that the substance is actually blood [56]. Dark substrates can pose considerable problems, due to the low contrast between the substrate and the fingermark, because of the high amount of incident light absorbed by the surface [56]. Other colors or patterns particularly similar to the stain can also cause issues for identification through

visual examination alone. Presumptive tests are therefore used as part of the current forensic workflow to indicate the presence of blood [55]. Despite a high sensitivity to blood, these wet chemical tests are not specific to blood and can generate false positives [55]. Wet chemical testing can also contaminate the stain, potentially having a detrimental effect on subsequent DNA analysis [57]. Previous and current research has therefore focused on alternate methods for the non-destructive identification of blood [54, 58–66].

Convictions can depend significantly on the ability to prove beyond reasonable doubt whether a bloody fingermark was deposited at the time a crime was committed or from a previous legitimate visit, as is often claimed by the defense team [39, 67]. There are currently no accepted analytical methods for reliably establishing a timeframe when a fingermark was deposited and speculation around age is subject to considerable error [68]. This is primarily due to the unreliability of previously proposed methods [69]. Successful identification of blood and estimation of the age of a blood-stained fingermark could provide the first indication to investigators as to when a crime was committed [70]. This could be especially beneficial if a blood-stained fingermark is the only evidence available.

It has been established that the color of a blood stain changes from red to brown over time [59]. This indicates that optical methods could be used to quantify the color of blood stains. This was first explored using the reflectance spectra, whereby the effect of environmental variables on the color of the blood stain was recognized [60]. Further research quantified absorption bands independent of the amount of blood present as a possible approach for age determination [61] or the use of a small spectral window [62].

Previous research has clearly established changes to the physical and chemical properties of blood over time [59]. An optimum technique requires a high selectivity to blood; a high level of sensitivity, even with diluted blood; and high levels of precision to determine the age of a blood stain or blood-stained fingermark in practice. One such method is the use of visible wavelength hyperspectral imaging (HSI). This was first reported for the detection and age determination of horse blood stains between 442 and 585 nm as proof of concept research [24]. The determination of age was obtained through linear discriminant analysis from data based on the progressive change of the absorption spectra over time as the composition of the blood stain altered. This approach used training and test datasets from the same blood stain in order to determine the age with high levels of accuracy. With different blood stains the accuracy was considerably lower, although this research demonstrated the potential of the method for establishing age estimation non-destructively. A similar method by a different research group has also been successfully demonstrated for the detection and age estimation of blood stains [71, 72]. The method proposed allowed for rapid, non-destructive presumptive blood stain detection as well as age estimation. Other research has explored forensic traces across a range of substrates [73]. Most recently a new blood stain identification approach was proposed based on the Soret γ band

absorption in hemoglobin [56], which indicated a higher sensitivity and specificity for the detection and identification of blood stains over previously proposed methods.

The need for a non-contact and non-destructive method for the determination of the age of a blood-stained fingermarkt is paramount. An ideal method should function across a practical range of ages of blood, have a high specificity to blood so as to prevent false positives and have a clear and accurate method for determining the age of a blood stain, so as to allow for reliable fingermark age estimations.

The visible wavelength HSI method described here meets all these requirements. We present the results of a novel application of visible wavelength HSI based on the absorption spectrum of hemoglobin between 400 and 680 nm for the non-contact, non-destructive detection, identification and age determination of blood-stained fingermarks on white tiles. False color scales are used to represent the age of the blood-stained specimen. A 30-day color scale is tested with nine blood-stained fingermarks of different unknown ages deposited on a single white tile to demonstrate the effectiveness of such a method for age determination. This work follows on from Cadd et al. [74–76] where hyperspectral imaging was used for the first time to detect, identify and visualize ridge detail in blood-stained marks across a wide range of substrates. The research described here demonstrates the potential of HSI, through successful non-destructive detection, identification, and age determination of this type of evidence.

3.6.1 Hyperspectral Reflectance Image Acquisition and Pre-processing

The HSI system used to obtain the results described in this chapter was similar to that detailed in Li et al. [56], consisting of a liquid crystal tunable filter (LCTF) coupled to a 2.3 megapixel Point Grey camera and a light source for scene illumination. The light source comprised of two 40 W LEDs; one violet giving an output at 410 nm and one white, giving an output between 450 and 700 nm. Control of the LCTF and image capture was performed using custom developed software written in C++ (Microsoft, USA). Images were captured between 400 and 680 nm with spectral sub sampling at 5 nm intervals, resulting in an image cube at 56 wavelengths for each scan. Spectra from the image cube were subsequently analyzed using custom routines developed in Visual Studio 2010 (Microsoft, USA) and Python 2.7 (Python Software Foundation, USA). The time required to acquire and process an image was approximately 30 s.

The hyperspectral reflectance measurements were made as follows. A reference image (R_0) was obtained using a blank substrate. This image was recorded in a 5 nm series of 56 discrete wavelengths between 400 and 680 nm. The sample image (R_s) was recorded at the same wavelengths under the same illumination conditions and integration time settings on the camera. The hyperspectral reflectance image (R) consisted of a data cube of 1280 × 1024 pixel values at 56 discrete wavelengths. From the reflectance images obtained, the pixels which satisfied the criterion were

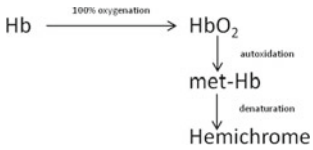


Fig. 3.9 Reaction of hemoglobin in blood stains. Reprinted from ref. [24] with permission from Elsevier

marked as black, whilst all other pixels were marked as white. This allowed regions of the image where the blood-stained fingermark was present to be identified, as well as a clear distinction of the ridge detail to be obtained. Additional information regarding sample image processing can be found in [56].

3.6.2 Criteria for the Identification of Blood-Stained Fingermarks

The presence of hemoglobin in blood dominates the blood reflectance spectrum in the visible region [59]. The spectrum contains a strong narrow absorption at 415 nm called the Soret or γ band with two weaker and broader absorptions between 500 and 600 nm known as the β and α bands [59] (Fig. 3.9). Due to the absorption in the blue part of the visible spectrum, the Soret band is responsible for giving blood its distinctive red color. Other red substances also absorb in the blue region of the visible spectrum between 400 and 680 nm. However, the width of these absorption features is typically much broader and also not centered at 415 nm. This forms the basis of the methodology to identify and discriminate blood-stained fingermarks and blood stains from other similarly colored substances. Further information is detailed in [56, 74–76].

3.6.3 Age Determination Methodology for Blood-Stained Fingermarks

The age of bloody fingermarks was explored through the effect of time on the composition of the fingermark. This has already been established as a potentially viable method for age determination, due to the numerous variables which affect fingermark composition over time [39]. This research explored the compositional changes that occur within a blood-stained mark using hyperspectral imaging. After deposition, specific chemical changes occur which result in a color change from bright red to dark brown. This is attributed to the complete oxidation of hemoglobin (Hb) to oxy-hemoglobin (HbO_2), which then auto-oxidises to met-hemoglobin (met-Hb) and denatures to hemichrome (HC) [59] (Fig. 3.9).

As this process occurs, the concentration of hemoglobin decreases, which can be observed in the visible spectrum through the decrease of the Soret band at 415 nm.

After HSI analysis, the absorption spectra were analyzed and the ratio of the peak of the β band at 525 nm to the trough at 550 nm (Fig. 3.10) was determined.

As blood ages, the height of the peak decreases whilst the trough increases and so the value of the ratio also decreases. The change in this ratio was used to produce false color Red-Green-Blue aging scales, as shown with ten false colored prints in Fig. 3.11, with the value of the ratio determining the values assigned to red, green and blue in the image. This is a simpler but less accurate method of blood age determination than the previously described method of Li et al. [23, 24] which used a statistical model based on linear discriminant analysis.

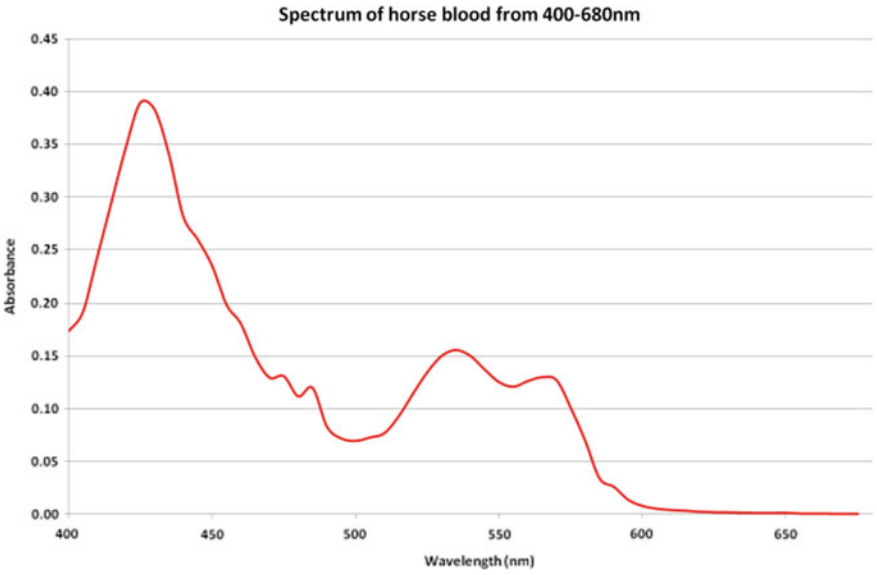


Fig. 3.10 Visible spectrum of blood from 400–680 nm. Reprinted from ref. [78] with permission from MDPI

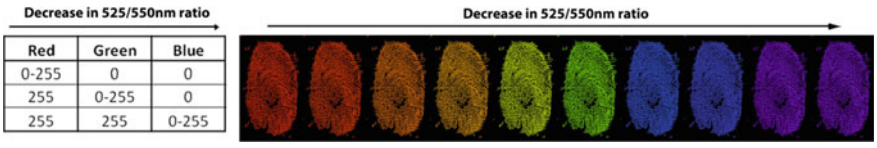


Fig. 3.11 Use of Red-Green-Blue colors to produce false color scales based on 525/550 nm ratio. Reprinted from ref. [79] with permission from MDPI

3.6.4 Age Determination of Blood Stained Fingermarks Using HSI

Detailed analyses of changes to the absorption spectrum over both twenty four hours and thirty days were carried out and separate false color scales were generated from both hyperspectral analyses. The thirty-day false color scale was then used to false color nine blood-stained fingerprints of unknown age based on their absorption spectrum obtained from hyperspectral analysis.

Blood-stained fingermarks were successfully detected using HSI for the full twenty four hours explored. Clear ridge detail was identified for all scans and the absorption spectrum analyzed to produce a false color scale, as shown in Fig. 3.12. Clear changes in color can be seen on almost an hourly basis over the twenty four hour period. This scale represents the changes that occur to the absorption spectrum over the twenty-four hour aging period, as shown in Fig. 3.13. The logarithmic conversion also shown demonstrates the clear relationship between the 525/550 nm ratio and time over this period.

Blood-stained fingermarks were also successfully detected and identified using HSI for the full thirty days explored. Clear ridge detail was identified for all scans, a selection of which is shown in seven day increments from deposition to twenty eight days in Fig. 3.14.

The level of clear ridge detail observable even after thirty days demonstrates the advantage of HSI over existing chemical methods, as not only can blood be conclusively identified, as opposed to only an indication obtained with presumptive tests; but ridge detail is both preserved and photographed for potential comparison in one step, even for blood-stained fingermarks which are not freshly deposited. Analysis of the absorption spectrum between 400 and 680 nm showed a clear decrease of the Soret band and the β and α bands between 500 and 600 nm over the aging period. This change forms the basis for the age estimation methodology. False color scales were produced to represent the changes to the 525/550 nm ratio over thirty days, as shown in Fig. 3.15.

From a simple visual examination of the nine blood-stained specimens alone, as shown on the left hand side in Fig. 3.16, it is very difficult to determine any significant differences that may allow for age estimations. Hyperspectral analysis however successfully detected and conclusively identified the ridge detail as blood. This data was then used to produce false color scales which give a clear visual representation of the different ages of the prints, as shown on the right in Fig. 3.16.

The DSLR images show minimal variation between the different ages of blood-stained fingerprints. However, using the false color scales, all freshly deposited prints can be easily distinguished due to the significant differences in color, such as prints five, eight, seven and three, which correspond to zero, one, three and six days respectively. After fourteen days, the variation in the composition of the blood-stained fingermarks is less, so the difference between the assigned false colors is smaller and harder to distinguish by eye. This is apparent for prints two, four and six, which are all shades of purple, despite varying by eleven days. The use of this false color

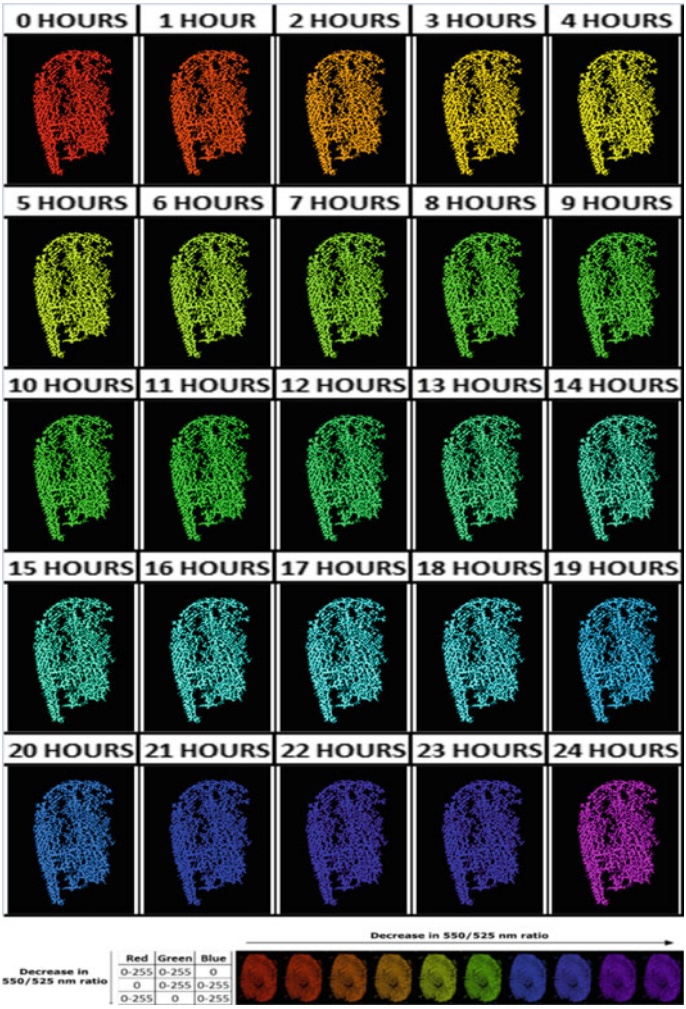


Fig. 3.12 A false color Red-Green-Blue aging scale generated from the ratio of the peak at 525 nm to the trough at 550 nm obtained through hyperspectral analysis of blood fingermarks over twenty four hours. Reprinted from ref. [79] with permission from MDPI

method for age estimations is therefore most effective for blood-stained specimens deposited within fourteen days, (and most so within the first twenty four hours), as the increased variation over the first seven days results in greater differences in the false color images produced.

The ability to determine age is greatly sought. The studies presented in this chapter have demonstrated the feasibility for such estimations, with the additional benefit of a false color aging scale to provide a clear, easy to distinguish, visual representation of the differences in ages between deposited specimens. Naturally, further research is

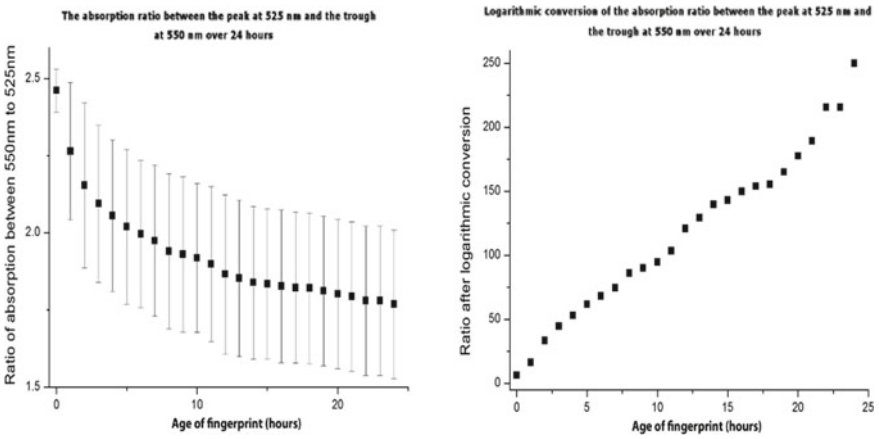


Fig. 3.13 Hyperspectral analysis was used to determine the effect of time on the absorption ratio between the peak at 525 nm and the trough at 550 nm (left) and the logarithmic conversion to show the relationship as a straight line (right) over the twenty-four hour aging period. Reprinted from reference [79] with permission from MDPI

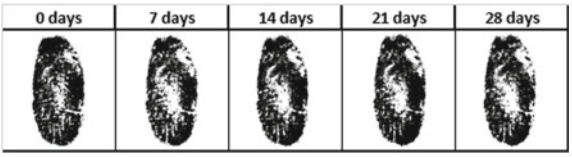


Fig. 3.14 Visible ridge detail for blood-stained fingermarks analyzed using HSI after zero, seven, fourteen, twenty one and twenty eight days. Reprinted from ref. [79] with permission from MDPI

required before such a methodology could be implemented into the existing forensic workflow, but the research detailed in this chapter has demonstrated that such a method exists, generates reliable results on white tiles, and, perhaps most importantly, is highly specific to blood. The key additional research which needs to be performed is to investigate firstly a greatly range of substrates and then the effect of environmental variables such as temperature and humidity on the aging process. These effects can in principle be parameterized and thus allow the creation of a robust and reliable model which can be used at crime scenes. The compatibility with prior use of existing CSI techniques also needs to be investigated.

3.7 Fingermark Age Estimations: Is There a Future?

In order to succeed in the challenging research field of fingermark aging, it is desirable and necessary to employ a robust technique, or combination of techniques, that

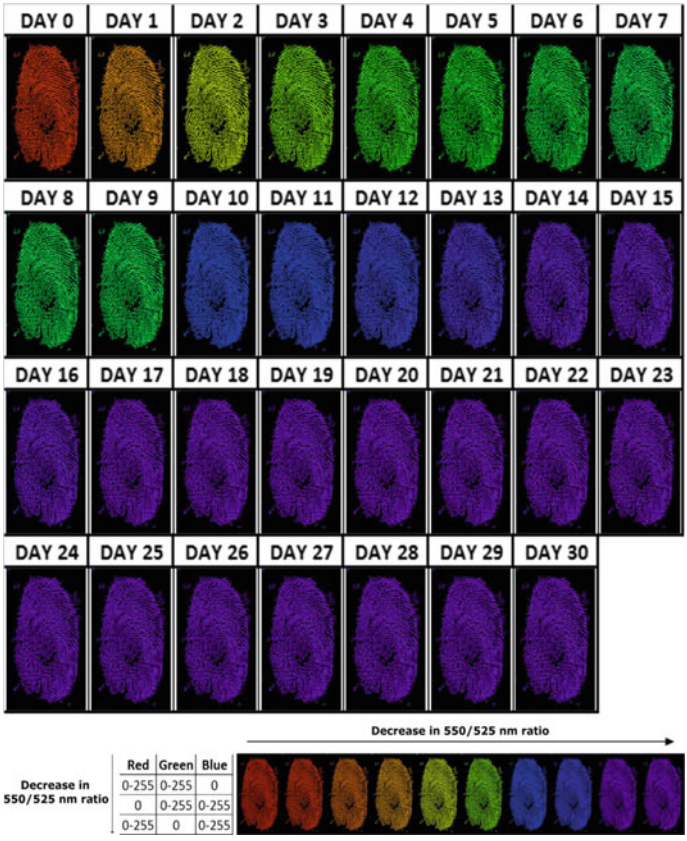
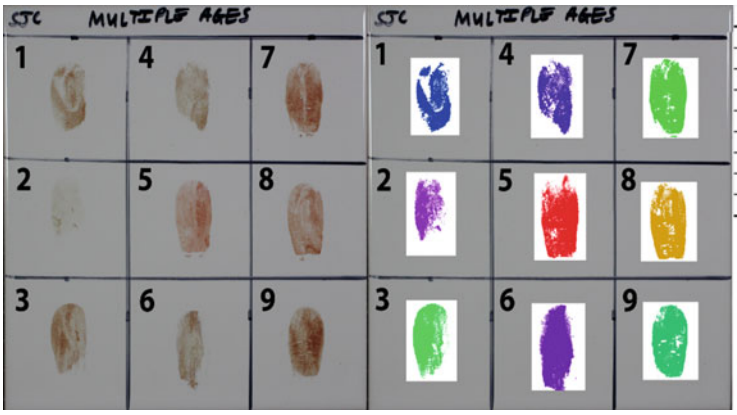


Fig. 3.15 A false color Red-Green-Blue aging scale generated from the ratio of the peak at 525 nm to the trough at 550 nm obtained through hyperspectral analysis of bloody marks over thirty days. Reprinted from ref. [79] with permission from MDPI

could overcome, and be able to explain, some or most of the variability exerted by environmental factors. Visual analyses, such as OP and HSI, can fulfill this objective because of their sensitivity to visual and chemical changes that take place during the aging process. Additionally, OP and HSI are able to collect topographical measurements that can be easily analyzed and interpreted.

After the first initial studies, further ideas arose on how to examine and develop models forfingermark aging patterns. Weyermann and Ribaux [77] describe that aging studies need to be focused on the kinetic properties of the evidence obtained and how to create a valid model that can be universally implemented. They note that although it is important to study the disappearance of features/full latent fingermarks as a function of time, it can be just as important to study the persistence of characteristics and even the alterations as a function of time. This means that although the disappearance of features can yield great knowledge, being able to determine



POSITION	AGE (Days)
1	13
2	28
3	6
4	22
5	0
6	17
7	3
8	1
9	9

Fig. 3.16 DSLR (left) and false color images manually colored from hyperspectral analysis (right) of blood-stained fingermarks based on thirty-day false color scale. Reprinted from ref. [79] with permission from MDPI

what does not change can yield relevant information as well. The struggle noted with the models presented is the ability for them to be reproducible and consistent under controlled conditions. Researchers have found it difficult to find features and trends that can be generalized to a large population of individuals. Overall, Weyermann and Ribaux developed research to help forensic scientists think outside the realm of what is already known in fingermark science to try to stimulate future ideas to address the challenges already facing the age determination of fingermarks [77].

From a technological perspective and based on the speed of engineering advances, OP and HSI could become operational in the field within a decade. However, these technologies will be useless to forensic scientists unless accompanied by a better understanding of degradation patterns and the effects of environmental and physical variables on age estimations. Based on past published data, from proof-of-concept studies in many cases, the time has arrived to upscale research and move beyond small, and at times unfunded, projects. This will be the only way knowledge on aging will pair with technological advancements.

As mentioned earlier, age estimations can provide, for the first time, valuable new information and intelligence about a crime beyond the possible presence of an individual. For this reason, more financial resources are needed to expand current research and ensure reliable results within a reasonable time span.

Great progress made in the field of fingermark aging gives hope for the future endeavors and research that is to come. There has been success in certain areas regarding latent and bloody fingermark age estimations; however, additional research effords need to be highlighted. There are seven key points to study moving forward:

- Identification of visual parameters and chemical decomposition products of aging fingermarks. To date, studies have gathered valuable knowledge on fingermark topography and composition; however being able to understand chemi-

cal intermediates and end-products can yield a better idea of how old the fingermark is by how far along it is in the reaction process.

- Scientists and researchers could delve deeper into deciphering and modeling the reaction kinetics of a (latent) fingermark. If the rates of decomposition can be determined for specific chemicals, the time intervals after deposition could be more easily estimated.
- Efforts should be made to study ratios of chemical compounds. Studies have shown how variable the quantities of different chemicals are; however, all these were conducted with a very small population of donors and/or samples. If the ratios of chemicals could be generalized using a more large-scale study, it would be easier to accurately extrapolate the age of the given print.
- One of the most important goals of future research is to carry out large-scale studies. It is very difficult for research findings to be generalized and implemented into real-world practice without enough data to produce accurate generalizations. A model, trend or any research cannot be certified as accurate, precise or reliable unless it can be completed by multiple researchers using multiple donor sets and reproduced on a large scale [39].
- The effect of donor, environmental, and substrate variables are critical areas of future focus. In a real scenario, (latent) fingermarks are not all aged under the same environmental conditions and on the same substrates. It has been shown that the location where the fingermark is deposited can have unique effects on its visual degradation. Much like in forensic entomology, if models can be created to map the acceleration or deceleration rates dependent on donor, environmental and substrate conditions, accurate age estimations of fingermarks would ensue.
- Translation of the statistical results into software algorithms: the software developed could be used in the field or in the office. A mobile app could automatically determine the age of the questioned fingermark based on input of a limited number of known (or estimated) environmental conditions (e.g. temperature and humidity) and certain visual degradation parameters (e.g. number of minutiae, ridge widths and heights). The software could even provide examples of reference fingermark images that could be easily correlated to the "questioned" deposition.
- Lastly, it is critical to continue collaborations and teamwork with different fields of science and technology, including statisticians in (latent) fingermark analysis. The only way for this forensic science to become more objective and more dependable in a court of law is to provide error rates, probabilities and other statistical models that make evidence more robust.

Overall, the ability to estimate the age of a fingermark might appear in the distant future. It is critical to start understanding the methods that will successfully help develop techniques to further this field of research. In terms of statistical evidence, researchers such as Kellman and Neumann [28, 78] have successfully began developing equations and models that can eventually be used as mathematical proof in court. However, these researchers are aware of the limitations of their work due to the heterogeneity of prints and continue to develop computerized systems that

can minimize and overcome the subjectivity that is currently present in the field of fingermark identifications.

In summary, many methods including visual, biological, chemical, and biochemical have shown to be valuable in providing partial knowledge to estimate the time of deposition of (latent) fingermarks. The move towards studying fingermarks in 3D seems to be the most logical and beneficial of paths moving forward. It is important to be aware that every field of research has limitations and to always draw conclusions with caution. Fingermark aging research is not an exception and needs to move forward by studying larger number of conditions and find commonalities among them, especially because there will be multiple types of environments, substrates and other undetermined factors influencing each fingermark's degradation process. In conclusion, this field of research will, in time, lead to answering the overarching question of how old a fingermark is, but it will take continued and groundbreaking research to develop suitable and creative techniques to solve such a challenging subject.

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Chapter 4
End User Commentary on Novel Technological Applications for Latent and Blood-Stained Fingermark Aging Studies

Aldo Mattei

As a matter of fact, since last century, forensic science borrowed technologies and methods from other scientific disciplines. In principle, this consolidated attitude has not to be regarded with a negative perspective. Being part of the broad science community should help forensic science to be more actively engaged with scientific methodologies and principles, as recommended by PCAST report: "Forensic Science in Criminal Courts: Ensuring Scientific Validity of Feature-Comparison Methods". Unfortunately, the trend over the decades in fingerprint science was to use these methods and principles, as formulated in the last century, in the daily routine, resulting in subjective assumptions without the necessary support of robust and reliable data.

By far, fingermarks and DNA analyses are the most widely forensic examinations leading to human identification, with a very high degree of admissibility as evidence in court.

The association between a fingermark found at the scene of crime and its potential donor mainly, or solely, originate from the morphological aspects of the trace, which have to find correspondence, within pre-determined tolerances, with a controlled print acquired from the donor. Currently, there are huge efforts among fingerprint community to standardize the steps of the methodology used to establish a correct association, and to populate with detailed sub-processes the so-called ACE-V method (Analysis, Comparison, Evaluation and Verification).

Moreover, as illustrated in the literature, fingerprints have the potential to reveal intelligence upon the donor habits, abuses, sex, DNA and gunshot residues. For the most, the acquisition of all these information comes through chemical analysis of the fingermark deposit and/or its contaminants. This type of intelligence could give to investigators and to criminal justice system a huge boost in the event reconstruction.

Nevertheless, the time of deposition of a fingermark remains an unsolved question for the vast majority of the cases. The time of deposition of the trace could not

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be practically considered as a valuable tool to avoid the erroneous attribution of a fingermark to a potential donor, reached on the bases of the friction ridge patterns and details.

In order to reduce erroneous inclusions, other avenues have to be followed. A re-consideration of the fingerprint evidence not anymore as a discrete variable, like a Boolean function, but as a *continuum*, which varies from exclusion to identification, will be the way forward for fingerprint science. Fingerprint comparison in the near future will be driven by probabilities and reference population data.

However, even under the hypothesis of negligible error rates in the comparison process between traces and reference prints, the time of mark deposition cannot be reliably estimated. Thus, fingerprint scientists could reasonably associate a person to a surface where the fingermark was recovered, but only in very rare cases, they are capable to prove to the trier of fact, when the fingermark residue was deposited.

Fingermark ageing arise as an issue when potential suspects had left their fingermarks at the scene and the trier of fact needs to discriminate between a legitimate accesses, from the one pertaining to the crime. In such case, fingerprint ageing becomes of paramount importance, but the answer to all these questions still needs to be found.

The main reason why this problem does not have a general solution yet, is due to the multiple factors governing the aging process, and their huge variations.

According to Girod, Ramotowski et al. five factors could be summarized, which have a relevant effect to the process: (1) donor sweat characteristics, i.e. intra-variability or inter-variability in the population, (2) deposition conditions, (3) type of substrate, i.e. material, roughness, porosity, electrical charge, coatings, (4) environmental conditions, i.e. variation of thermodynamic quantities, coupled with mechanical effects due to atmospheric agents, (5) visualization techniques used to reveal the fingermarks.

Because of the aforementioned reasons, at present, a robust and reliable technique does not exist allowing the determination, with an acceptable degree of approximation, the age of a fingerprint residue.

In literature, the publication of the very few aging studies started in the 1960s. From the early studies, probably the most complete is the one carried on by Holyst, published in 1987, lately largely used for the purpose.

In addition, some case reports were published, describing ad hoc experiments, carried on in very specific conditions, with limited consideration for the chemical and physical properties of the sweat, resulting in a very limited applicability in general cases. Some of them conducted to the conviction of the suspects, while some others were not considered to this respect.

Whenever the evidential value of a fingermark is disputed by a legitimate access to the scene, specific aging studies could be conducted by a very careful consideration of all parameters considered by Girod, Ramotowski et al. using the same visualization technique applied at the scene and/or in the laboratory. In order to evaluate correctly the most variable quantities, such as sweat composition and fingermark quality, depletion series and multiple donors should be used in the experiment. In any case, the results must be correctly evaluated, without discarding any possibility.

In a murder case occurred in Bologna, Italy, where the author carried out the investigation, a fingermark, among others, was developed with black powder on the frame of a door in the house of the deceased.

Later, a fingermark comparison was carried on, resulting in the association of the mark with the younger son of the victim. According to the very limited information usually available to the forensic department, the association was considered as a legitimate access, and therefore of no evidential value. Nevertheless, the prosecutor office contacted again the laboratory few months later: the outcome of the investigations proved that the younger son of the victim had no more relations with his family since almost ten years before the incident. Thus, the question "*How old was the fingermark found on the frame?*" was formulated by the prosecutor office; in this case, ageing assumed a high relevance in the case, even if only at a later stage.

The scene of crime was accessed again. A careful inspection of the surface of the frame was carried on, in order to analyze the nature of the surface and to start to design an ageing experiment. Eventually, during the examination, the Carabinieri verified that the mark was slightly indented in the transparent coating of the frame. Every further attempt to enhance the mark with the black powder and to lift it with acetate adhesive tapes was successful. The mark was left in the coating when the coating itself was still fresh, at the time of the last painting of the frame of the door.

The above-mentioned case has the purpose to demonstrate: (1) how ageing of fingermarks occurs in the daily work of fingerprint laboratories, (2) how difficult is to establish standard procedures to address the matter, (3) the need to be cautious to generalize the assumption that a good quality mark is necessary fresh.

Thus, we would recommend to subject matter experts to be reluctant in expressing any opinions in court, based solely on their experience, on the estimation of age of fingermarks. Some inferences could be made only in particular circumstances, whenever substantiated by verified hypotheses through a robust verification, which implies the strict application of a scientific methodology.

According to the literature available, also the quality of the mark could not be considered as the sole determinant for age estimation. In fact, cases of good quality marks have been reported after 30 years after their deposition.

More recently a series of studies has been published on fingermark aging which considers chemical composition of fingermarks. Ageing studies has been conducted since 1995 considering the degradation processes occurring in fingerprint chemical compounds, i.e. oxidation processes and decomposition reaction due to time.

More recently, Wyermann and Girod conducted a more comprehensive study started in 2013, which targeted lipids and their transformation due to time factors as a potential source of ageing measure of fingermark deposit. Among more than a hundred of others, ten lipids were selected with statistical techniques, as more stable in each donor (lower intra-variability) and the decomposition process has been analyzed by means of chemical techniques, as GC/MS, FTIR also combined with chemical imaging, resulting in a vast series of publications between 2013 and

2016. Nevertheless, blind trials conducted, showed reliable age estimation only in less than 2/3 of the examinations.

In parallel, the successful forensic application of the study of fingermark proteins by MALDI-MS have opened the field to the application of proteomics also for ageing researches. More recently, proteomic studies, recently published by Oonk et al. in 2018 seems to have identified a dating signature, by chemical analysis of fingermark residue obtained by LC-MS, which is composed by 5 proteins. This subset of proteins has been reported as time dependent and, with a certain rate, detectable after bodily fluids contamination.

In the near future, the analytical approach would certainly produce robust and reliable results. Even though this type of analyses requires high level of expertise, dedicated and costly equipment, are (partially) destructive of the sample, and they seem not to be applicable at the scene of crime, the science seems really promising, compared to other techniques. Moreover, it could be applied after visualization techniques.

However, at this point in time, from the perspective of the end user, it is unrealistic to invest efforts and resources to develop an in situ ageing technology. Developing crime lab technologies to be used remotely seems of greater importance in order to achieve a robust and reliable methodology, which allows the fingerprint expert to determine the age of the marks, especially in case the fingerprint evidence is challenged by the defendant at a later stage, i.e. claiming a legitimate access. This technology, operated in laboratory by adequately trained (forensic) scientists, should be used only in these particular circumstances.

Even though some authors theorized the opportunity to develop ageing technologies capable to be deployed at the scene of event (Wei et al. 2016), it would be more productive to concentrate the efforts in the development of a reliable technique for laboratory operations, whilst, as a latter improvement, to explore the feasibility of a mobile application.

Because of the above-mentioned reasons and in consideration of the status of the art of the research, it seems to be unrealistic to propose the inclusion of the ageing determination as a streamlining step to prioritize fingermark visualization at the scene of crime or to speed up database searches.

The main unsolved forensic problem is the age determination of the fingermarks, not the search of the unknown marks in the available databases. Currently AFIS search is very fast, robust and accurate at the point that if any ageing technique would be applicable to the vast majority of the cases, it should be applied after the comparison process.

Besides the examination of the variation in time of chemical properties and the degradation processes of the chemical constituents of the fingerprint residue, similar approach could be valuably applied to the study of the variation of physical properties of the fingermark, i.e. the thickness, the continuity of the ridges, or their response to the electromagnetic radiation. The two different techniques proposed by the authors of this chapter, the Optical Profilometry (OP), that visualize and describe variations in the topography of ridges, and the visible wavelength Hyperspectral Imaging (HSI), that measures spectral changes in blood-stained fingerprints, are two promis-

ing technique. The physical principles underpinning these two researches are robust and reliable. However the maturity of the two methods appears to have different technology readiness levels.

Optical Profilometers (OPs) are instruments capable to analyze very small surfaces in two (2D) or three dimensions (3D). OPs are currently used for the study of surface roughness of materials. The main advantage of 3D OP is due to the fact that is a contactless, three-dimensional technique, thus preserves the fingermark uncontaminated. The visualization of the trace is obtained by a detector (i.e. a CCD camera), that collect the signal coming from the inspected surface. By means of white light interferometry, it is possible to determine the height and widths of ridges and creases of the fingermark onto the surface.

The proposed approach consists in the realization of a validated mathematical model, which could allow the estimation of the age of a finger deposit on the determination of the changes occurred due to time, and other parameters considered in the model, to the physical properties measured with the OP.

The research has—in principle—all the factors needed to succeed. However, the state of the art needs further studies and investments. In particular, models need to be tested on various types of surfaces (i.e. the most common found at the scene of event). In fact, the persistence of the fingermark, for a given donor, is highly dependent on the nature of the surface. Moreover, two major questions/issues need to be raised. Firstly OP measures not only the fingermark on the surface, but also the roughness of the surface itself. Thus, would the technique be only suitable for applications on smooth surfaces? The second issue is related to the time needed to obtain the topographical image of a surface. Considering an area of 100 cm^2, given the considered technology, and if used without the pre-application of CSI or crime lab enhancement techniques, the time needed to obtain the data is over 10 h. This time lapse does not allow the application of the technique at the scene of event.

Concerning fingermarks contaminated with blood, they assume a particularly high evidential value in the contest of a violent crime. Traditionally, fingermarks produced by blood contamination, or produced by subtraction of blood in a blood pool has been regarded as almost coeval to the crime. However, this assumption may lead to erroneous conclusions. With laboratory trials, it is possible to demonstrate how fingermarks produced with other contaminants, mainly greasy substances (i.e. olive oil and hand cream) lately wiped with blood, assume the appearance of fingermarks produced by blood contamination. To this respect, chemical imaging techniques could be very suitable methods to determine whether blood is exactly located only on fingermark ridges or in other parts of the trace, thus making a more robust evaluation of the so-called “activity level” of the trace.

In any case, ageing of blood traces could solve disputes, which might arise in investigative scenarios, where the determination of the age of blood could avoid any claim of previous deposition of fingermarks by the defendant. With this perspective, hyperspectral imaging (HSI) is a technique that can be successfully applied to forensic scenarios, including the detection, identification and age estimation of bloodstains. The simple principle is based on the recording of images at series of different wave-

lengths. The spatial information at the various wavelengths is summed up in a "third dimension" containing all the single spatial data at the different wavelengths.

HSI applications might use all the electromagnetic spectrum. Typically, the wavelengths range from the ultraviolet (UV) to the infrared (IR). Multiple configurations can be designed: a simple one, used for this purpose, consists of a tunable filter applied in front of the detector, selecting the broad band light directed on the surface in steps of five nm from 400 to 680 nm.

One of the major advantages of this technique consists of its capability to determine that the "reddish" substance at the scene of event, or on the item, is actually blood.

In fact, the bloodstain identification approach based on the Soret γ band (415 nm) absorption in hemoglobin, demonstrated a high sensitivity and specificity for the detection and identification of bloodstains, compared to other methods.

The HSI method is contactless and non-destructive, highly specific for blood, and allows also the determination of the age of the traces in an interval of time of 14 days, with a great discrimination within the first 24 h.

The research explored the compositional changes that occur within a blood-stained fingerprint, which result in a color change from red to brown, due to the complete oxidation of hemoglobin (Hb) to oxy-hemoglobin (HbO_2) and, finally to hemichrome (HC).

After HSI analysis, the absorption spectra have been analyzed. A ratio of the peak of the β band at 525 nm to the trough at 550 nm has been selected as determinant for the age. The change in this ratio has been assumed for the establishment of a false color scale, which is very easy to interpret. At present, this research could be implemented in some real scenarios, because of the high specificity to blood and its non-destructive characteristics. However, for the use on real cases, some hypotheses need to be made on temperature and humidity of the environment where the trace was located, in order to calibrate the model, assuming the use of the same type of substrate.

The challenging research field of fingermark dating needs a concerted effort between a combination of techniques, detecting variations of the chemical and physical properties of fingermarks in time. Other factors, which are of paramount importance in the ageing process, are: (1) the gradients of the thermodynamic quantities, (2) exposure to air fluxes and atmospheric agents, (3) the nature of the surface.

Visual analyses, such as OP and HSI, should be promising techniques. OP seems to be still at an early stage, while an attempt to apply HSI could be made whether the blood mark age determination is necessary in a particular investigation.

From the end user perspective, in order to have in the future some reliable and robust ageing methods, investments to fund research are desirable, even if the field of application will be limited to the laboratory and/or to some specific subcategories.

The future researches need to consider the chemical decomposition products of fingermarks, their kinetics and, eventually, chemical compound ratios in statistically relevant samples of population. The effect of environmental factors and substrates could be subsequently determined, generalizing models designed within confined boundaries.

The interpretation of the tested and calibrated data could be eased by user-friendly interfaces, which can allow the forensic scientist to present the evidence with the support of robust and reliable methodologies.

In conclusion, the possibility to determine the age of a fingermark is still limited at present. Any conclusion in court regarding the age estimation should be done underlining limitations to the assumptions made. Nevertheless, the research in this field should be encouraged and supported, given the paramount importance of this information to the correct evaluation of the fingermark evidence by the trier of fact.

Chapter 5
Bioanalytical Advancements in the Reliable Visualization and Discrimination of Bodily Fluids

James Gooch, Alvaro Varela Morillas and Nunzianda Frascione

Abstract Body fluids are an important form of biological trace evidence that can be used to substantially inform many aspects of criminal investigations; fluids such as blood, semen, and saliva can provide investigators with more information about the specific nature of an offence and associate individuals with a crime via DNA profiling. However, many of the techniques currently used to locate and identify body fluids left at crime scenes suffer from low specificity, sample destruction and lengthy operation times. As a result, many members of the forensic and academic communities are working together towards the development of new rapid, sensitive and specific body fluid analysis methods. This chapter initially provides an overview of the fluid detection and attribution strategies currently employed within routine forensic casework and their associated weaknesses. Next, a selection of spectroscopic and molecular techniques that show the most promise as replacements for traditional fluid testing strategies, along with the merits and limitations of each method, are described.

5.1 Introduction

Biological fluids represent one of the most important types of trace evidence in forensic casework. The detection and identification of fluids such as blood, semen and saliva may be of enormous evidential value to a case, both in terms of investigative information and personal identification. This importance is reflected by the fact that over 90% of the items submitted each year to the Evidence Recovery Unit of the Metropolitan Police Service (the largest police force in the United Kingdom) are tested for the presence of biological fluids.

Such fluids may allow investigators to learn more about the specific nature of an offence. For example, establishing the presence of seminal fluid at a scene may help determine if a crime should be investigated as a sexual assault, rather than a physical

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assault. The location and morphology of biological fluid stains can also be used to provide information on the actions and movements of individuals/objects during an offence. In fact, the entire discipline of forensic Blood Pattern Analysis (BPA) is dedicated to the reconstruction of events based on the shape and directionality of bloodstains found at crime scenes.

Body fluids also have significant value in forensic casework as a major source of DNA. The analysis of genetic material contained within fluids can allow investigators to draw definitive scientific links between individuals and an offence with great reliability. During the 2016/17 calendar year, DNA profiling technologies were used to match over 31,000 DNA samples recovered from crime scene to profiles present in the UK National DNA Database (NDNAD) [1]. These technologies have been expanded upon in recent years to also provide phenotypic information directly from fluid samples. Assays based on the analysis of single nucleotide polymorphism (SNP) markers have now been developed for the prediction of an individual's externally visible characteristics (including hair colour, eye colour and geographic ancestry) and may be used identify potential fluid donors that cannot be matched via traditional DNA typing [2].

However, obtaining such sources of DNA can be a significant challenge, as fluids left behind during a criminal offence are often difficult to detect. Many fluid traces are deposited at scenes in extremely low amounts (often in microlitre or lower volumes) or may be heavily diluted as a result of mixing with other body fluids or liquid substances. Most fluids (including semen, saliva, urine, vaginal secretions and sweat) are also transparent, making them difficult to observe against black or dark-coloured backgrounds. In some instances, the perpetrators of a crime may attempt to remove, wash or disguise fluid stains in order to avoid detection [3]. An array of techniques currently used to detect latent body fluid stains can be found in Table 5.1.

The first stage of the biological fluid detection process involves the visual inspection of an item searching for any areas of potential fluid staining. This inspection requires a considerable amount of time, as every area of the item must be examined in detail by naked eye and/or using optical microscopy. However, because of the challenges listed above, it is still possible for forensic scientists to miss vital fluid evidence during this process. As a result, several tools have been developed to facilitate fluid detection. Alternate light sources (ALS) are devices that may be used to visualise some latent fluids by providing greater contrast between a stain and the surface on which it is deposited. These devices produce fixed wavelengths of ultraviolet, visible or infrared radiation that can be absorbed by specific molecules present within biological fluids. In some instances (particularly in the case of blood), this absorption will cause stains to appear darker than their surrounding background.

However, some molecules present in semen, saliva and urine, may become excited by ALS radiation and emit their own light (at a longer wavelength than the original source), making the fluids fluorescent [4]. Some of the chemical tests used to establish the identity of a fluid (which will be discussed in detail later in this chapter) also have the ability to detect areas of latent staining.

Several high-profile UK criminal cases have recently served to highlight the potential miscarriages of justice that can occur as a result of missed fluid evidence. The

Table 5.1 Techniques currently used by forensic investigators for the detection of latent biological fluid stains

Body fluid	Test	Type	Notes	References
Blood	ALS	Spectroscopic	Blood appears as dark spots when exposed to ALS wavelengths between 300 and 900 nm	[4]
	Luminol	Chemical	Haemoglobin acts as a catalyst in a reaction between luminol and an oxidant (typically H_2O_2) to produce chemiluminescene	[5]
	Bluestar®	Chemical	A proprietary luminol formulation containing H_2O_2 and NaOH, offering greater detection sensitivity and signal strength	[6]
	Fluorescein	Chemical	Reduced fluorescein (fluorescin) is oxidised by H_2O_2 in the presence of haemoglobin, producing fluorescence at 521 nm	[7]
	Hemascein®	Chemical	A commercial preparation of fluorescein, NaOH and zinc with greater shelf life than traditional fluorescein formulations	[8]
	Lumiscene®	Chemical	A proprietary mixture of both luminol and fluorescein, producing chemiluminescence at a wavelength of 525 nm	[9]
Semen	ALS	Spectroscopic	Choline and Flavin-conjugated proteins cause semen to strongly fluoresce under UV and visible excitation	[4]

(continued)

Table 5.1 (continued)

Body fluid	Test	Type	Notes	References
	Acid Phosphatase Press Test	Chemical	Moist paper sheets are applied to items and treated with a solution containing α-napthyl phosphate and brentamine salts, which turns purple in the presence of acid phosphatase	[10]
Saliva	ALS	Spectroscopic	Fluoresces weakly upon UV and visible excitation. Molecules responsible for emission have yet to be identified	[4]
	Phadebas® Press Test	Chemical	Commercially available paper sheets impregnated with starch microspheres that can be placed over items. Digestion of spheres by salivary amylase causes a blue dye to be released	[11]
Vaginal fluids	None	N/A	No methods currently accepted for use in forensic casework	N/A
Urine	ALS	Spectroscopic	Exhibits weak fluorescence at multiple ALS wavelengths	[4]
Sweat	None	N/A	No methods currently accepted for use in forensic casework	N/A

most prominent of these involved the racially motivated murder of British teenager Stephen Lawrence by a group of individuals in 1993. As part of the initial investigation, clothing seized from six men accused of the murder was subjected to two forensic screenings (one in 1993 and another in 1995) for the presence of blood, both yielding negative results. However, in a re-examination of all forensic evidence as part of a cold case review started in 2006, a previously missed blood spot was discovered dried into the fibres of a jacket belonging to one of the suspects. Identified through DNA profiling as having originated from Stephen Lawrence, this blood (along with other forensic evidence) was used to convict two of the perpetrators in 2012, almost 20 years after the murder had taken place.

In some cases, characterising the type of body fluid present is equally as important as detection. For example, in an instance of alleged sexual assault in which DNA from a suspect is found on the surface of the victim's skin, a defendant may argue that this DNA may have been indirectly transferred from saliva present on surface of their hands. However, if that DNA is proven to have instead originated from seminal fluid, evidence that a sexual assault did take place will be significantly stronger. Although not always possible, DNA evidence is likely to be much more useful to the courts if its origins can be attributed at the 'source-level' (i.e. to a specific biological fluid) [12].

Many assays are available to the forensic scientist for the purpose of body fluid attribution. A list of fluid identification strategies currently used within forensic casework is reported in Table 5.2. These tests vary significantly in their mechanism of action as well as their specificity, sensitivity and ease of operation. Assays are often classified as to whether they are 'presumptive' or 'confirmatory' in nature. Presumptive tests are those only able to indicate that a fluid may be present or establish the absence of a fluid from a sample. These assays cannot be used to conclusively identify a fluid species as they may also positively react in the presence of non-body fluid substances or cross react between different fluid types [3]. In contrast, confirmatory tests are able discriminate fluids with a relatively high degree of certainty. However, presumptive tests are usually inexpensive and rapid to perform and are therefore often initially applied to a suspected fluid stain to determine whether or not a secondary confirmatory test is required [5]. However, not all fluid identification assays have the ability to visualise latent stains. Those relying on the generation of colorimetric products (which cannot be observed against most coloured surfaces) or necessitating analytical instrumentation to observe positive signals usually require stains to be located through traditional visual searching methods before tests can be undertaken. As previously mentioned, some fluid discrimination tests may also be used for the purposes of stain detection. The luminol assay for the identification of blood relies on iron present within haemoglobin to catalyse the oxidation of the luminol molecule by hydrogen peroxide. A positive reaction results in the chemiluminescence emission of luminol at a wavelength of 431 nm. Consequently, forensic investigators are able to disperse the luminol reagent over surfaces in which blood is suspected to be present to rapidly locate areas of fluid staining (albeit presumptively) through the production of blue-coloured light [5]. However, not all fluid identification assays have the ability to visualise latent stains. Those relying on the generation of colorimetric products (which cannot be observed against most coloured surfaces) or necessitating analytical instrumentation to observe positive signals usually require stains to be located through traditional visual searching methods before tests can be undertaken. A list of fluid identification strategies currently used within forensic casework may be found in Table 5.2.

Table 5.2 Techniques currently used within forensic casework for the attribution of body fluids

Body fluid	Test	Type	Specificity	Notes	References
Blood	Hemastix®	Chemical	Presumptive	Plastic test strips coated at one end with H_2O_2 and Tetramethylbenzidine (TMB) impregnated filter paper. Haemoglobin catalysis causes paper to change colour from yellow to blue/green	[13]
	Kastle-Meyer	Chemical	Presumptive	A colourless reduced form of Phenolphthalein is oxidised by H_2O_2 in the presence of haemoglobin to produce an intense pink colouration. Usually applied to a swab or filter paper that has been rubbed over the surface of a suspected stain	[14]
	LMG	Chemical	Presumptive	Oxidation of colourless Leucomalachite Green to Malachite Green by H_2O_2 occurs in the presence of haemoglobin, resulting in a blue-green colour	[15]
	ABAcard® HemaTrace®/Hexagon OBTI/SERATEC® HemDirect®	Immunological	Presumptive	Dye-labeled haemoglobin-antibody complexes are formed after the addition of extracted blood-stains to an immunochromatographic cartridge. These complexes migrate through the device to a 'test' zone and are captured by a second anti-haemoglobin antibody. A coloured line produced as a result of dye aggregation is used to indicate a positive result on the surface of the cartridge	[16–18]

(continued)

Table 5.2 (continued)

Body fluid	Test	Type	Specificity	Notes	References
	RSID™—blood	Immunological	Presumptive	Immunochromatographic cartridge targeting red blood cell protein Glycophorin A. Demonstrated to be specific for the presence of human blood	[19]
	Takayama	Crystal	Confirmatory	Heating of a bloodstain treated with pyridine and glucose under alkaline conditions causes the formation of pink, needle-shaped crystals made of pyridine ferroprotoporphyrin. Crystals may be observed using normal bright-field microscopy	[20]
	Teichmann	Crystal	Confirmatory	Brown hematin crystals are formed as a result of heating dried bloodstains in the presence of glacial acetic acid and a halide (usually a chloride salt), which can be observed microscopically	[21]
	Microscopy	Visual	Confirmatory	The visual identification of red blood cells, white blood cells and fibrin within a liquid fluid sample may be used to confirm the presence of blood	[3]
Semen	Acid Phosphatase	Chemical	Presumptive	A reagent containing α-napthyl phosphate and a diazonium slat (such as Brentaine Fast B or Black K) is applied to filter paper or swabs that have been rubbed over a suspected semen stain. Hydrolysis of α-napthyl phosphate by seminal acid phosphatase produces a purple azo dye	[22]

(continued)

Table 5.2 (continued)

Body fluid	Test	Type	Specificity	Notes	References
	ABAcard® p30/ Biosign® PSA/ SERACTEC® PSA Semiquant	Immunological	Presumptive	Immunochromatographic cartridges targeting the seminal fluid protein prostate specific antigen (also known as p30). Have been shown to cross react with urine, vaginal fluid and breast milk	[23–25]
	RSID™—Semen	Immunological	Presumptive	Immunochromatographic cartridges targeting the seminal fluid protein semenogelin	[26]
	Microscopy	Visual	Confirmatory	The microscopic identification of spermatozoa may be used to confirm semen presence. Cell staining reagents may be utilised to facilitate observation. These include the Christmas tree and hematoxylin-eosin cytological stains	[3]
Saliva	Phadebas®	Chemical	Presumptive	A propriety product (produced in tablet or paper form) containing starch microspheres labelled with and insoluble blue dye. Spheres are digested in the presence of salivary α-amylase, releasing the dye, which can be identified visually or by using a spectrophotometer at 620 nm	[27]
	RSID™—Saliva/SERATEC® Amylase	Immunological	Presumptive	Immunochromatographic cartridges targeting the salivary protein α-amylase. Have been shown to cross react with urine, semen and breast milk	[28, 29]

(continued)

Table 5.2 (continued)

Body fluid	Test	Type	Specificity	Notes	References
	SALIgAE®	Unknown	Presumptive	A commercially available colorimetric assay for the detection of saliva. The exact mechanism of the test has not been disclosed. Stain extracts are added to a glass vial containing the SALIgAE® solution, which turns yellow within 10 min in the presence of salivary α-amylase	[30]
Vaginal Fluids	None	N/A	N/A	No methods currently accepted for use in forensic casework	N/A
Urine	DMAC	Chemical	Presumptive	Suspected urine stains are extracted onto filter paper using distilled water before a drop of 0.05–0.1% para-dimethylaminocinnamaldehye (DMAC) solution is applied. Ammonia, released in the breakdown of urea by the enzyme urease, reacts with the DMAC compound, resulting in a pink colour change that can be seen after 30 min	[31]
	Uritrace®	Chemical	Presumptive	Lateral flow cartridges containing reagent strips impregnated with picric acid. Creatinine from urine extracts added to the cartridge reacts with the acid, forming creatinine picrate and causing the strips to turn from yellow to orange	[32]
	RSID™—urine	Immunological	Presumptive	Immunochromatographic cartridge targeting the Tamm-Horsfall protein	[32]
Sweat	None	N/A	N/A	No methods currently accepted for use in forensic casework	N/A

5.2 Recent Advances in Body Fluid Visualisation and Discrimination

Many of the assays currently used for the detection and attribution of body fluids have been routinely employed in forensic casework for several decades. In fact, the first use of the Kastle-Meyer reagent for the identification of bloodstains was reported in the late 1920s [14]. However, in recent years, forensic science (and its use within the legal system) has changed significantly. Increases in the DNA profiling capabilities of forensic service providers, as well as mounting pressure on scientists to assure courts of the reliability of analytical tests, has led many members of the forensic and academic communities to question whether or not current body fluid testing strategies are still fit for purpose.

Rapid profiling technologies (such as the IntegenX® RapidHIT® or ParaDNA® systems) are poised to revolutionise forensic DNA analysis by providing full genetic profiles from crime scene samples in less than two hours [33]. However, casework turnaround times are still likely to be constrained by the lengthy fluid detection and identification processes that must be conducted prior to DNA testing. This is largely due to the fact that both processes cannot be conducted simultaneously, as there are no methods currently available to locate latent stains and confirm fluid identity at the same time. In cases where the type of body fluid likely to be present on an item is unknown, investigators may have to perform multiple fluid discrimination tests in sequence on the same stain. This not only increases analysis times, but also reduces the amount of DNA available for downstream profiling applications, as portions of a stain are consumed with every test [34]. Presumptive assays currently used for body fluid attribution are also limited in their evidential usefulness, due to issues of low analytical specificity. The potential for false positive or false negative results to occur (as a result of cross reaction or test inhibition respectively) in tests such as the Kastle-Meyer reagent is well known to the courts [35].

Therefore, any expert witness opinions based on the outcomes of presumptive assays are likely to be contested by defence barristers, who may provide other equally plausible alternative explanations for the test results.

Consequently, significant research efforts have been made in the development of new rapid, sensitive, specific and non-destructive body fluid detection and identification strategies. These efforts are often undertaken as part of collaborative partnerships between academia, police organisations and/or private forensic science providers. Techniques that have shown the most promise as replacements for conventional body fluid testing methods are summarised below.

Infrared Spectroscopy Infrared (IR) spectroscopy involves the analysis of a sample based on its interaction with IR radiation. This radiation is traditionally divided into three distinct regions based on relative distance from the visible portion of the electromagnetic spectrum: near- (800–2500 nm), mid- (2500–25,000 nm) and far-IR (25,000–10^6 nm). As each of these regions is able to induce different measurable vibrations in covalently bonded atoms and groups, IR spectroscopy may be used to identify unknown compounds by providing information on their molecular structure.

Consequently, IR spectroscopy has become extensively employed in forensic analysis (e.g. inks, paints, fibres and hairs) [36]. Investigations into the identification of biological fluid samples using IR spectroscopy have also been undertaken and have mainly focused on the use of near (NIR) and mid-Infrared (MIR) radiation.

NIR is the most energetic form of IR radiation. It is used to detect overtones (electronic transitions of more than one energy level) and combinations (vibrational signals of two identical bonds that appear split due to Fermi resonance), which can provide structural information on bonds involving hydrogen [37, 38]. NIR spectroscopy may be considered particularly amenable as a method of body fluid attribution as it is non-destructive, rapid, capable of analysing samples through plastic or glass containers and requires little-to-no sample preparation.

Furthermore, advances in instrument miniaturisation have allowed the development of portable NIR devices, which are already being explored for the analysis of biological fluid stains directly at crime scenes [39, 40]. In one recent study, a handheld NIR device was used to identify bloodstains on a series of surfaces commonly encountered in forensic casework. This device was able to successfully differentiate blood from several blood-like substances (including red wine, ink, tomato sauce, coffee fake blood, food colouring, paint and beetroot juice) in up to 94% of cases [39]. As NIR spectra are often disadvantaged by the presence of broad, overlapping peaks that cannot be distinguished by the naked eye, this study also used a series of chemometric models for the automatic interpretation of spectroscopic signals. Such an approach may allow for the operation of the device by police staff or scenes of crime officers that have not been trained in IR spectra interpretation.

Conversely, MIR spectroscopy may be used to detect changes in both the vibrational and rotational states of bonds between carbon and other atoms as a result of IR excitation. MIR analysis has also been investigated as a method of fluid attribution, with previous studies able to identify patterns of spectral peaks characteristic of macromolecules present in blood, semen, saliva, urine and vaginal secretions [41, 42]. However, all studies conducted so far have relied on the use of benchtop MIR instrumentation, preventing the deployment of this technique directly at crime scenes. Another disadvantage currently associated with MIR-based biofluid analysis includes the prominent spectral bands observed as a result of water molecule presence, which may potentially mask detectable protein analytes. Furthermore, MIR (as well as NIR) spectroscopy techniques are currently only able to determine the identity of a fluid and cannot be used to locate areas of latent fluid staining.

Raman Spectroscopy While most spectroscopic techniques involve observing the amount of radiation absorbed, reflected or emitted by a sample, Raman spectroscopy relies on measuring the inelastic scattering of light to identify molecules. In this technique, samples are irradiated with low-energy monochromatic laser beams, causing the scattering of incident photons. A small percentage of these photons will exhibit a change in frequency in an effect known as a Stokes (or anti-Stokes) shift [36]. The wavenumbers and intensities of these shifts may be used to provide valuable information on the molecular structure, intermolecular interactions and internal environment of a sample. Such information can now be obtained from femtolitre or picogram

amounts of liquid and solid samples respectively [43]. As a result, Raman spectroscopy has been extensively studied for the detection and identification of body fluid traces within forensic casework.

The first investigation into the use of Raman spectroscopy for the identification of biological fluids involved the characterisation of blood deposited onto glass slides, as well as dried blood particles present on tape lifts [44]. In this study, four laser wavelengths were successfully used to establish blood presence by monitoring the vibrational spectrum of differently oxygenated haemoglobin molecules. However, this technique was unable to distinguish human blood from that obtained from other mammalian species. This research has been significantly expanded upon in many studies for the identification of multiple body fluids (including peripheral blood, semen, saliva, sweat, vaginal fluid and menstrual blood) based on their Raman signature (Fig. 5.1) [45].

In fact, a number of studies have recently demonstrated the extraction of phenotypic information, such a donor age, sex and race directly from fluid samples through Raman spectroscopy [46–48]. Recent efforts to increase the sensitivity of Raman-based fluid attribution methods have also been made through the use of surface enhanced Raman spectroscopy (SERS), in which samples are placed on the surface of metallic sheets or nanostructures to enhance Raman signal strength [49].

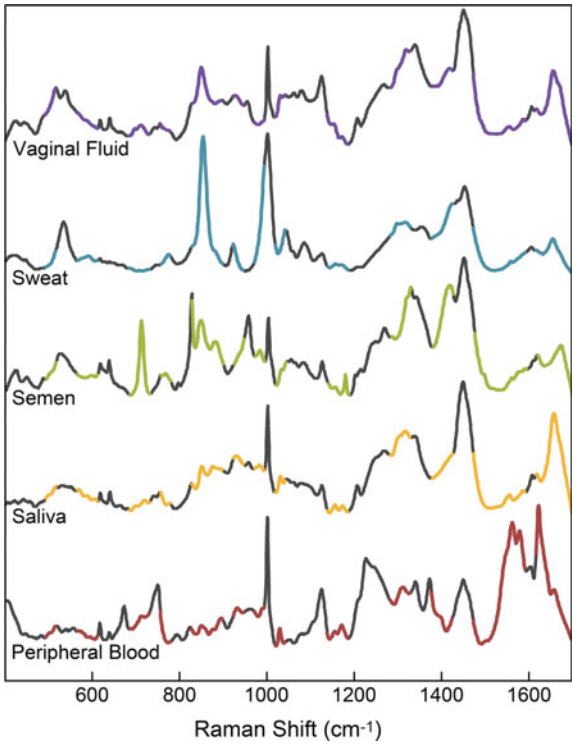

Fig. 5.1 Pre-processed mean Raman spectra of dry traces of peripheral blood, saliva, semen, sweat, and vaginal fluid exhibit unique profiles, which reflect their biochemical compositions and allow for confirmatory identification. The most informative variables for classification selected by genetic algorithm are shown in colour, while the variables discarded from the model are in black. Reproduced with permission from [45]

This approach is likely to be extremely useful in cases where trace amounts of fluid are present, or for use with portable Raman spectrometers, which are considerably less sensitive than laboratory-based instruments.

One major disadvantage associated with Raman spectroscopy is the potential for signal interference from the auto-fluorescent background surfaces on which a fluid is deposited. The intensity of these fluorescence emissions, which are produced through the excitation of substrates by the laser light used to affect photon scattering, is often several orders of magnitude larger than that of Raman signals. Such interference may prevent the identification of a body fluid stain by masking portions of its vibrational signature [50]. However, this issue may be solved by utilising a radiation source from the NIR region of the electromagnetic spectrum.

Hyperspectral Imaging Originally designed for the purpose of remote sample sensing, hyperspectral imaging (HSI) combines regular imaging and spectroscopy for the simultaneous collection of spatial and spectral information from an object. In this technique, reflectance, absorbance, fluorescence or Raman spectra from tens to hundreds of narrow wavebands across the electromagnetic spectrum are obtained for each pixel in a two-dimensional image [51]. These spectra are then used to construct a three-dimensional data structure, comprising two spatial dimensions (x and y) and one wavelength dimension (λ), known as a hypercube (Fig. 5.2). Hypercubes are able to provide users with a complete image for every collected wavelength, as well as a contiguous reflectance spectrum for each image pixel. As such, HSI is emerging as a powerful tool for probing the chemical composition of forensic samples, including biological fluid traces.

A series of studies have recently demonstrated the exceptional performance of HSI techniques in the detection and identification of human bloodstains [52–55]. In these studies, hyperspectral images taken in the visible and NIR regions of the electromagnetic spectrum were used to rapidly identify blood deposited on a range of coloured surfaces, whilst also allowing them to be differentiated from other blood-like substances. Furthermore, the use of HSI as a potential method for the estimation of bloodstain time since deposition was also established [53, 56]. Subsequent studies

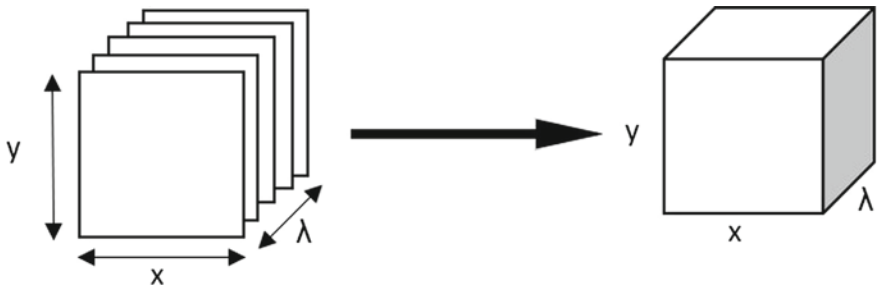

Fig. 5.2 An illustration of hypercube construction. Image data from two spatial dimensions (x, y) is combined with spectral data from a series of narrow wavebands (λ) to form a three-dimensional data structure

have sought to extend upon this research by utilising HSI for the identification of other body fluids, including semen, saliva and vaginal fluid [57, 58].

The introduction of HSI to the field of forensic analysis remains relatively recent, therefore extensive validation studies to assess method performance under differing environmental conditions are likely to be required before this technique can be deployed for the analysis of biological fluids in routine forensic casework. Such conditions may include the variability of ambient lighting at crimes scenes (especially those located outdoors), which is known to exert significant influence over the quality of collected HSI spectra [51]. Other challenges to the use of HSI within forensic analysis include the interpretation of complex spectra from mixed or contaminated samples. This is especially important in the testing of biological fluids, which are often found as part of multi-fluid mixtures [51].

RNA Profiling In order to fulfil its biological function, each type of cell present within a tissue will exhibit a distinct pattern of gene expression. These patterns can be characterised by measuring the type and/or abundance of RNA transcripts expressed by the cell. As biological tissues contain multiple cellular components, much research has gone into the forensic discrimination of body fluids based on their overall transcriptomic 'signature'. This process is known as RNA profiling. Although several types of RNA molecule have been investigated for fluid identification purposes, most studies have focused on the analysis of messenger RNA (mRNA) [59].

One of the main advantages of mRNA profiling over traditional body fluid identification techniques is that multiple types of body tissues can be tested for simultaneously in a single multiplex reaction, as shown in Fig. 5.3. This includes fluids that cannot be identified by conventional means, such as menstrual blood and vaginal fluid [59]. The analysis of multiple mRNA markers per body fluid also ensures a high degree of assay specificity [60]. A list of core mRNA markers used in fluid attribution assays, as well as the proteins with which they are associated, may be found in Table 5.3.

Initial investigations into forensic mRNA profiling involved the use of the reverse transcriptase-polymerase chain reaction (RT-PCR) for the identification of epithelial cells within menstrual blood stains [73]. This technique was subsequently improved upon to allow the analysis of markers from multiple fluids through the incorporation of a capillary electrophoresis (CE) detection step [63]. Since then, RT-PCR with CE has largely been accepted as the standard method for mRNA-based fluid attribution in most forensic laboratories. However, a large collaborative exercise recently carried out by the European DNA Profiling group (EDNAP) and EUROFORGEN Network of Excellence has also demonstrated the potential of massively parallel sequencing (MPS) technologies for the targeting of fluid-specific transcripts [69].

These validation studies have cemented mRNA profiling as the most likely candidate to replace conventional body fluid identification methods. In fact, a number of forensic laboratories, including the Netherlands Forensic Institute (NFI) and the Institute of Environmental Science and Research (ESR) in New Zealand have already begun to utilise mRNA profiling methods within their operational casework [70]. However, mRNA profiling has yet to be adopted on a global scale. This is likely due

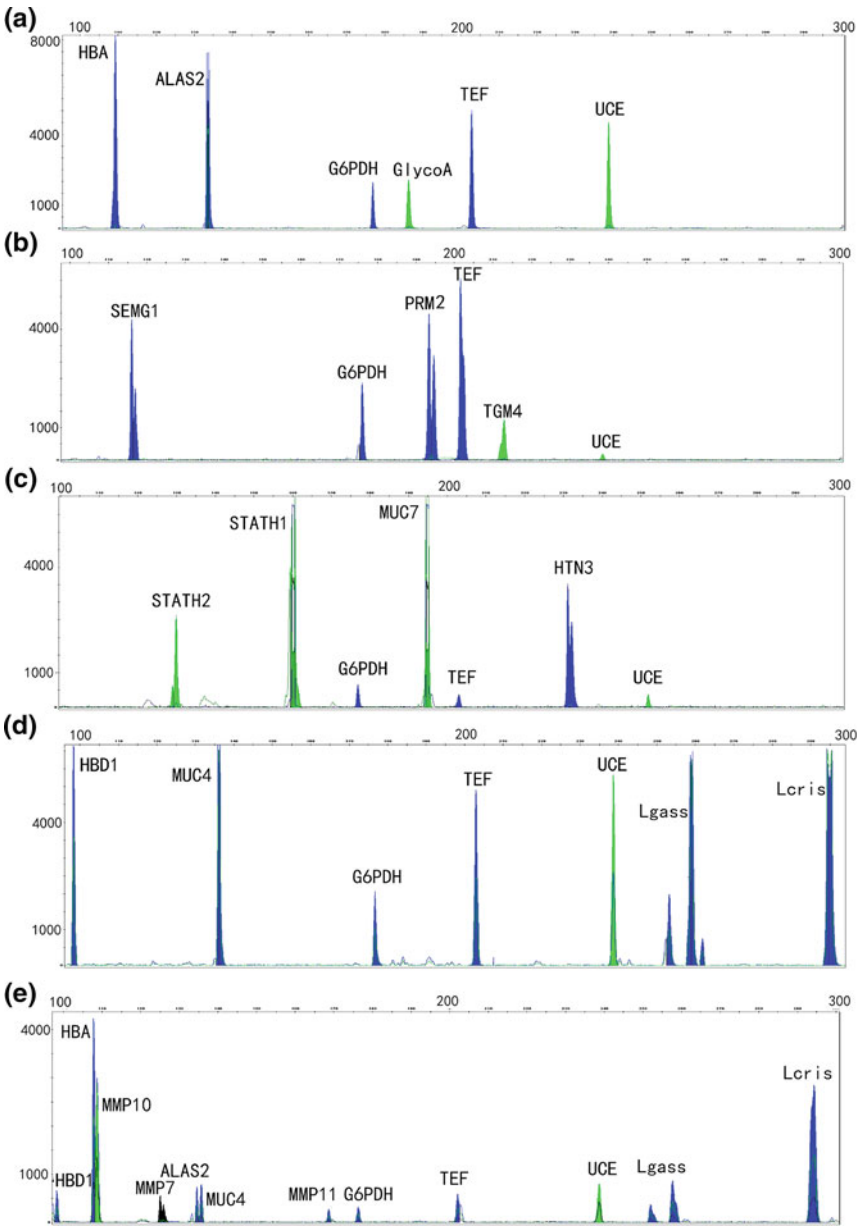

Fig. 5.3 Electropherograms of the mRNA multiplex profiles of blood (**a**), semen (**b**), saliva (**c**), vaginal secretions (**d**) and menstrual blood (**e**). The mRNA was marked at the top of the peak. No marker expression signal was detected in blank (rt-cDNA) samples. Peaks at 125 bp and 134 bp were dye-blobs. Reprinted from Song et al. [113] with permission from Elsevier

Table 5.3 Genetic markers commonly used in mRNA multiplex assays for the identification of body fluids

Body fluid	Gene name	Associated protein and protein function	References
Blood	ALAS2	Delta-aminolevulinate Synthase 2—Erythroid-specific mitochondrial protein	[61]
	ANK1	Ankyrin 1—helps attach membrane proteins to cellular cytoskeleton	[62]
	SPTB	Spectrin Beta Chain—role in cell membrane structure and organisation	[63]
	CD3G	T-Cell Surface Glycoprotein CD3 Gamma Chain—adaptive immune response	[64]
	CD93	Cluster of Differentiation 93—transmembrane receptor glycoprotein	[65]
	AMICA1	Junction Adhesion Molecule Like—protein present on Leukocyte membrane	[66]
Semen	PRM1	Protamine 1—replaces histones in spermatogenesis, compacting sperm DNA	[67]
	PRM2	Protamine 2—replaces histones in spermatogenesis, compacting sperm DNA	[67]
	TGM4	Transglutaminase 4—critical reproduction protein secreted by the prostate	[62]
	SEMG1	Semenogelin 1—predominant semen protein. Forms seminal gel matrix	[62]
	SEMG2	Semenogelin 2—predominant semen protein. Forms seminal gel matrix	[62]
	KLK3	Kallikrein 3—known as prostate specific antigen. Breaks down seminal matrix	[62]
Saliva	HTN3	Histatin 3—salivary protein responsible for antimicrobial activity in oral cavity	[68]
	HTN1	Histatin 1—salivary protein responsible for antimicrobial activity in oral cavity	[63]
	STATH	Statherin—prevents precipitation of calcium phosphate salts within saliva	[68]
	PRB3	Proline Rich Protein BstNI Subfamily 3—bacterial receptor in parotid saliva	[68]
	PRB4	Proline Rich Protein BstNI Subfamily 4—bacterial receptor in parotid saliva	[62]
	PRH2	Proline Rich Protein Haell Subfamily 2—provides protection for dental enamel	[69]
	PRB1	Proline Rich Protein BstNI Subfamily 1—bacterial receptor in parotid saliva	[68]
	MUC7	Mucin 7—gel forming protein serving lubricating functions in the oral cavity	[70]

(continued)

Table 5.3 (continued)

Body fluid	Gene name	Associated protein and protein function	References
Vaginal Fluids	CYP2B7P1	Cytochrome P450 Family 2 Subfamily B Member 7 Pseudogene 1	[71]
	DKK4	Dickkopf WNT Signaling Pathway Inhibitor 4—role in embryonic development	[71]
	FAM83D	Family with Sequence Similarity 83 Member D—regulates cell proliferation	[72]
	CYP2A6	Cytochrome P450 2A6—responsible for oxidation of nicotine and cotinine	[72]
	CYP2A7	Cytochrome P450 2A7—functionally inactive cytochrome P450 enzyme.	[72]
Menstrual Blood	MMP10	Matrix Metalloproteinase 10—involved in breakdown of extracellular matrix	[73]
	LEFTY2	Left-right determination Factor 2—associated with endometrial bleeding	[74]
	MMP7	Matrix Metalloproteinase 7—involved in breakdown of extracellular matrix	[63]
	MMP11	Matrix Metalloproteinase 11—involved in breakdown of extracellular matrix	[73]
	SFRP4	Secreted Frizzled-related Protein 4—may regulate adult uterine morphology	[74]
Skin	LCE1C	Late Cornified Envelope 1C—precursor of stratum corneum layer of epidermis	[75]
	CCL27	C-C motif Chemokine 27—role in attracting lymphocytes to cutaneous sites	[75]
	IL37	Interlukin 37—protein suppressor of immune and inflammatory responses	[75]
	SERPINA12	Serpin Family A Member 12—modulates insulin action in adipose tissues	[72]
	KRT77	Keratin 77—responsible for epithelial structure in skin and sweat glands	[76]
	COL17A1	Collagen Type XVII Alpha 1 Chain—critical role in strengthening skin tissue	[76]

to the forensic community's lack of general consensus on how to present mRNA profiling evidence in court. Unlike the analysis of short tandem repeats (STRs) in DNA profiling, there is currently no established framework for the statistical reporting of mRNA results that incorporates a probabilistic measure of uncertainty [77]. However, research in this area is currently underway [78].

A major challenge to the uptake of mRNA profiling by forensic providers is the complex laboratory procedures required for transcriptome analysis. This includes the transcription of mRNA into cDNA by RT-PCR and the removal of contaminating genomic DNA from samples via DNAse treatment [79]. Many authors have also

expressed concern over the reliability of methods used to quantify total amounts of RNA, which if not carried out correctly, can result in the production of non-specific profiling artefacts through target over-amplification [79, 80].

One disadvantage of mRNA profiling compared to other recent advances in body fluid testing is that it can only be used for the purposes of fluid attribution and not detection. All fluid stains subjected to mRNA analysis must still be located using the time-intensive searching methods previously listed [81]. However, the use of mRNA profiling in forensic casework is still likely to result in a reduction in fluid analysis times, as both RNA and DNA from stains can be extracted simultaneously [82]. The stability of RNA molecules within body fluid stains is also highly contested. In order to meet the changing demands of protein synthesis, RNA transcripts are rapidly degraded by cells through a number of enzymatic pathways. As a result, these transcripts typically have much shorter half-lives in comparison to their DNA counterparts (although some specific transcripts have been shown to be more stable than others) [83]. However, several studies have also demonstrated the sufficient recovery of RNA from a range of aged and environmentally compromised body fluid stains [84, 85].

DNA Methylation Profiling The unique patterns of gene expression analysed as part of mRNA profiling are regulated in the cell through a number of biochemical pathways. 'Epigenetic' changes in expression are those that do not depend on the nucleotide sequence of DNA, but instead involve alterations to the packaging and structure of the DNA molecule itself. These alterations include histone modification, chromatin structuring and DNA methylation [86]. The latter of these has recently become of great interest to the forensic community, due to the established variability of methylation patterns within body tissues.

In mammalian DNA, methylation is achieved almost exclusively by the addition of a methyl group ($-CH_3$) at the 5-carbon position of a cytosine base to form 5-methylcytosine. This process is mediated by DNA methyltransferase enzymes and usually occurs at 'CpG sites', where cytosine nucleotides are directly followed by guanine residues in the linear sequence of bases. Unmethylated CpG sites are often grouped together towards the regulatory region of genes in areas known as 'CpG islands', where they exercise control over transcription activities [86].

In recent years, several forensic applications based on the analysis of DNA methylation have been proposed. Many studies have demonstrated that methylation patterns are altered in response to various lifestyle and environmental stressors, including diet, physical activity, stress and chemical exposure [87]. As a result, DNA methylation analysis has been suggested as a possible method for the genetic differentiation of monozygotic twins, a feat that cannot be accomplished by standard STR typing [88]. Some research groups have also begun to investigate the use of methylation analysis for the estimation of an individual's chronological age directly from biological samples [89].

However, one of the most promising applications of methylation analysis to forensic investigation is as a method of discriminating biological fluids. Much like RNA expression, DNA methylation patterns have been shown to exist in a tissue-dependent

manner [90]. Since initial work on epigenetic-based fluid attribution began in 2011 [91], a range of differentially methylated regions (DMRs) specifically associated with forensically relevant tissues, including peripheral blood, semen, saliva, vaginal fluid and menstrual blood, have been identified. Whilst significantly more research has been undertaken in the identification of fluid stains through mRNA profiling, DNA methylation analysis may be considered an attractive option for use in forensic casework due to the potential for analysing methylation and STR loci in the same reaction, allowing body fluid attribution and DNA profiling processes to be carried out simultaneously [91].

Many techniques have been employed for the detection of fluid-specific DNA methylation. These can be separated into three distinct categories based on the type of DNA pre-treatment methods used prior to analysis: methylation sensitive restriction enzyme digestion, protein/antibody affinity binding and sodium bisulfite conversion [92]. However, as each of these methods suffers from either analytical sensitivity or specificity, no assay has been universally recognised by the forensic community as the standard method of fluid methylation analysis. Techniques able to detect many epigenetic markers as part of large multiplexes (Fig. 5.4) generally require significant

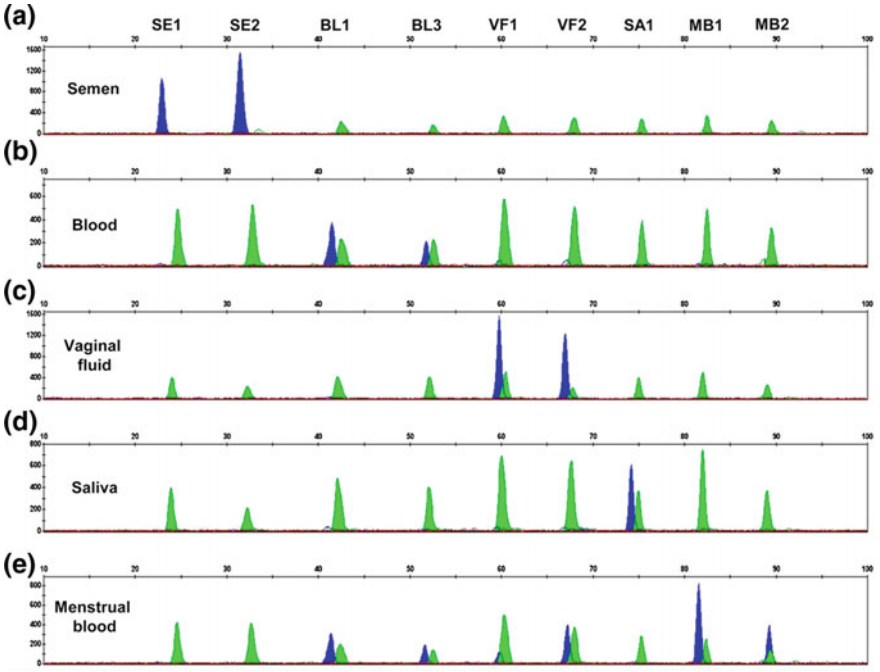

Fig. 5.4 Representative electropherograms of body fluid identification using multiplex methylation SNaPshot; (**a**) semen, (**b**) blood, (**c**) vaginal fluid, (**d**) saliva, and (**e**) menstrual blood. SE1, SE2, BL1, BL3, VF1, VF2, SA1, MB1 and MB2 represent cg17610929, cg26763284-138d, cg06379435, cg08792630, cg09765089-231d, cg26079753-7d, cg09652652-2d, cg18069290 and cg09696411, respectively. Reprinted from Lee et al. [114] with permission from Elsevier

amounts of high-quality input DNA in order to function efficiently, which is likely to be problematic in the profiling of low volume body fluid traces typically found at crime scenes [93]. Conversely, sensitive methods that may be used to analyse sub-nanogram amounts of DNA are currently restricted in the number of methylation markers that can be detected in a single reaction, thereby increasing the potential for erroneous results [93]. However, MPS technologies, such as the illumina® MiSeq™ platform, have recently shown great promise for the sensitive and reliable multiplex analysis of epigenetic markers [89, 94].

The technical challenges associated with current DNA methylation assays are not the only factor preventing the large-scale adoption of epigenetics-based fluid attribution methods by forensic laboratories. Some studies have demonstrated that tissue-specific methylation markers may be susceptible to change as a result of individual ageing and/or exposure [95]. A more extensive validation of previously reported fluid attribution markers is likely to be required before DNA methylation technologies are accepted for use within operational casework. The Israeli company Nucleix has already conducted one attempt at the commercialisation of a tissue-specific methylation assay; DSI-Semen™ is a 5-loci methylation multiplex for the identification of seminal fluid, analysed using methylation-sensitive restriction enzyme digestion coupled with PCR amplification [96]. However, this product appears to have been later removed from the market.

Metagenomic Analysis The human 'microbiome' refers to the population of microbes that reside within or on the surface of human bodily tissues. Such microbes, which include bacteria, archaea, fungi, protists and viruses, have been reported to be present in the body in amounts ten times greater than that of human cells (although this number is highly disputed, with some authors claiming the ratio to be closer to 1:1) [97, 98]. Recent studies have shown that despite the variation in microbiomes between individuals (as well as in the same individuals over time), particular body sites tend to be inhabited by distinct microbial communities [98]. As a result, these communities may serve as the basis for the forensic attribution of body fluid stains based on their metagenomic 'signature'.

The most prevalent method of identifying the type of microbes present within a specific community is through the targeted sequencing of the 16S ribosomal RNA gene. This gene is generally present in all microbiota but contains a number of hypervariable regions, which may be used for the purpose of species discrimination [99]. Recent improvements in sequencing technologies (as well as reductions in sequencing costs), has also led some forensic researchers to consider the use of whole-genome sequencing for the characterisation of certain microbiomes [100]. Although not yet applied to field of biological fluid attribution, such techniques may be able to provide investigators with greater levels of taxonomic discrimination, as well as an indication of relative species abundance, by analysing the entire genome of each type of microbe present within a population.

So far, only a small selection of the body fluids commonly found at crime scenes have been able to be identified through metagenomic analysis. Furthermore, those microbial markers that have been reported for the purpose of attribution may only

be used to determine the body site from which a fluid has originated, but not the identity of the fluid itself. For example, successful detection of the bacterial species *L. crispatus* or *L. gasseri* within a sample may be able to establish that a fluid is either menstrual blood or vaginal fluid, but cannot distinguish between the two, as they both originate from the vaginal cavity [101]. Other tissues for which body site-specific markers have been identified include saliva, faeces and skin [59]. Some authors have also stated that microbial profiling is unlikely to be suitable for the analysis of 'sterile' fluids, such as blood and semen, in which bacteria is not usually present in large quantities [102].

Other challenges currently associated with this approach include the potential for a fluid's microbial signature to change after it has been deposited at a crime scene. Changes in the relative abundance of particular microbe species as a result of growth or degradation may make it difficult to identify fluids left at scenes over prolonged periods of time [59]. Inter-individual variation also makes the selection of standardised metagenomic markers problematic, as even some widespread microbe species have been shown to be absent in certain individuals [103]. An established overlap between the microbiome of humans and other mammalian species also means it is unlikely that microbial-based attribution methods will be able to determine whether or not body fluid samples are of human origin [104].

Biosensors Biosensors are a group of devices capable of turning biological interactions into observable signal outputs. Usually consisting of a specific biological recognition element (such as an enzyme, antibody or nucleic acid sequence) directly coupled to physicochemical transducer, biosensors have had great impact in a number of disciplines in which the accurate detection and quantification of an analyte is essential [105]. Whilst already routinely utilised in clinical diagnostics, environmental monitoring and pharmaceutical discovery, biosensors have yet to fully enter the field of forensic analysis.

Several studies have recently demonstrated the exciting potential of biosensor technology for the location and identification of body fluid traces deposited at crime scenes [106–109]. In these studies, a number of molecular sensing reagents were developed which, upon interaction with intra-fluidic targets, indicate fluid presence through fluorescence emission. The spray dispersal of these reagents across the surface of large items may allow for the specific and non-destructive localisation of fluid staining areas in real-time. Furthermore, it may eventually be possible to combine several of the biosensing molecules previously reported within a single multiplex assay (which produces specific wavelengths of light depending on the type of fluid present) for the simultaneous visualisation and attribution of fluid stains. Employment of these reagents within routine forensic casework may lead to a significant reduction in the labour and time expense associated with current manual stain search and identification strategies.

To date, three different sensing mechanisms have been evaluated for this purpose (Fig. 5.5). Initial investigations focused on the use of fluorogenic substrates, a group of biosensors traditionally used within clinical diagnostics to determine the concentration of protease enzymes present in biological samples. These substrates

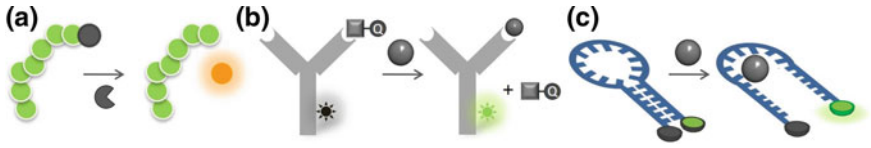

Fig. 5.5 Demonstration of different sensing mechanisms explored for body fluid analysis; **a** a terminally labeled fluorogenic substrate is digested by catalytic enzymes, releasing a fluorescent by-product; **b** analyte-induced conformational change upon antibody binding changes donor-acceptor proximity, resulting in a wavelength shift/fluorescent signal; **c** a fluorophore-quencher pair in an initially quenched oligonucleotide hairpin (e.g. aptamer) is separated upon binding, restoring fluorescence. Adapted from Ref. [115] with permission from The Royal Society of Chemistry

are comprised of fluorophores that are initially quenched as a result of coupling to a specific amino acid sequence. This sequence is recognised and subsequently cleaved by a target protease, resulting in the separation of the peptide-fluorophore bond and the restoration of fluorescence emission. As enzyme presence differs between tissues types, these substrates may find application in the forensic discrimination of body fluids stains based on their proteolytic activity. So far, three fluid-specific fluorogenic substrates have been reported; one commercially available assay that was applied to the detection of seminal fluid [106] and two custom-synthesised substrates for the analysis of semen and saliva [107, 108]. Each of these substrates was able to successfully identify fluids deposited on a range of surfaces relevant to criminal investigation. Importantly, all sensors were found to have no detrimental effects on downstream DNA profiling processes.

Two assays based on fluorescent immunosensing have also been explored for the purpose of body fluid identification. The first of these involved the use of an anti-glycophorin A antibody conjugated to a highly fluorescent quantum dot (QDot) nanoparticle. In the presence of human blood, emission from the QDot was quenched in a concentration-dependent manner [110]. However, with positive reactions indicated through the absence of fluorescence, it is unlikely that this 'turn-off' sensor could be used to visualise blood staining areas. An attempt to improve upon this assay was subsequently made in the design of a 'turn-on' displacement immunosensor targeting the seminal fluid protein prostate specific antigen (PSA). Here, the emission of an anti-PSA antibody-QDot complex was first quenched through the moderate binding of a peptide PSA analogue. This peptide was labeled with a dye able to absorb photons emitted by the QDot when placed in close proximity. In the presence of the native PSA protein, the analogue is competitively displaced, preventing quencher absorption and resulting in increased fluorescence emission at 625 nm [109]. An advantage of this assay format is that it may be easily adapted towards other body fluid targets for which antibodies are commercially available. Moreover, the use of immunological-based recognition also ensures a high degree of assay specificity, limiting the potential for sensor cross-reaction.

Research into the development of biological fluid 'aptasensors' is also currently underway. Aptamers are single-stranded DNA or RNA sequences that may be raised in vitro to bind to virtually any given target, including proteins, small molecules,

cells and tissues [111]. Whilst analogous to antibodies in terms of binding specificity, aptamers possess a number of key advantages over their protein counterparts, such as increased stability, cost effectiveness and production efficiency. As a result, aptamers may make attractive recognitions moieties for use within fluid-biosensing assays [112]. A range of electrochemical, mass-sensitive and optical transduction platforms may be used in the construction of aptasensors; however, the latter of these is likely to be the most amenable to the forensic detection of latent biological fluid stains.

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Chapter 6
End User Commentary on Bioanalytical Advancements in the Reliable Visualization and Discrimination of Bodily Fluids

Chris Gannicliffe

With the increasing sensitivity of DNA analytical methods such as DNA17 and DNA24, and the implementation of advanced software to help interpret complex mixtures of DNA from multiple persons, forensic science has never been better placed to address the '*who*'. However the challenge now for the forensic practitioner is that the sensitivity of DNA analysis is so great that it far outstrips the capability of the more rudimentary methods that identify the '*what*'. The screening methods used to initially search a crime scene or items at the laboratory so that stains can be visualised, and the subsequent tests used to confirm the body fluid type in the stains, have in many ways not evolved significantly over several decades. As the authors describe, current searching methods rely heavily on meticulous visual searching of the crime scene or items in the laboratory, supplemented by low magnification stereomicroscopy and alternative light sources, to identify stains that can then be tested chemically or microscopically to confirm the body fluid. Where the stains are more visually challenging to find, the tests instead rely on chemical screening methods that involve directly spraying chemicals on the suspect surface (for example a carpet or item of clothing), or chemically testing filter paper that has been speculatively rubbed over or blotted onto a garment.

Current chemical or catalytic-based body fluid tests used in casework are relatively insensitive, and often cross-react with other body fluids or household materials. The sequential nature of the testing regime can require a lot of the stain material, threatening the potential for later DNA analysis, and finally, current body fluid testing methods are constrained by, in some cases, an inability to be utilised as a broader searching method (for example urine testing), and the lack of any current test that can reliably identify menstrual blood, vaginal fluid or nasal secretions.

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This review of the advancement of visualising and discriminating body fluids is therefore to be warmly welcomed. The techniques outlined by the authors range from those in the relative developmental infancy of 'proof of concept', through to those already successfully implemented in casework but now looking to evolve into a more mainstream method that can be integrated into current laboratory protocols. Some offer opportunities as screening methods to assist searching a large area of a textile garment for example, whilst others offer possibilities to assist in confirming the identity of stains that have already been located. All however offer exciting possibilities for the future.

As the authors highlight, a significant advantage of vibrational spectroscopy (FTIR and Raman) methods is that they adopt equipment already in use at many forensic laboratories for the analysis of paint, glass and fibres. There are however several challenges to overcome to progress the technology to casework—fundamental to those is the fact that body fluids are complex biochemical mixtures whose constituents may vary between donors causing problems in reliable body fluid identification. Casework samples are typically aged, degraded or comprise mixed body fluids, and whilst conventional enzymatic methods generally give a binary positive/negative result, with poorer quality samples vibrational spectra may show alterations in relative peak intensities or noisy baselines that hinder interpretation. To progress this technology would firstly require significant investment in method development, moving from the current neat body fluid samples used in proof of concept studies to casework-type samples of mixed body fluids and degraded samples. The methodology would then need thorough validation, demonstrating that the technique is fit for purpose and understanding its limitations, and finally there would need to be development of statistical applications to assist in Raman spectral interpretation. The development of portable analytical equipment described in the review, moving the method from the laboratory bench to the crime scene, might be critical to the successful application of the methodology in a casework setting.

The development and application of hyperspectral imaging in the forensic field faces many of the same challenges as vibrational spectroscopy—as the authors identify, this is still in its early stages, and while it might offer potential for searching for body fluid stains using visible/NIR scanning, extensive validation studies are needed to progress the technology to real-world situations. The authors correctly identify some immediate challenges for future research to address, but in addition the future methodology has to overcome current difficulties with scanning crime scenes that comprise assorted substrates with differing light-scattering qualities, as well as practical problems such as streamlining the current protracted protocol.

Of all the advancements in body fluid identification discussed by the authors, the one that is the most mature, in that it has already begun to be incorporated into routine casework, is mRNA profiling. As the review describes, the immediate attraction of mRNA profiling is that a multiplex approach can be taken, and vaginal fluid and menstrual blood, which cannot be characterised by existing chemical or enzyme-based methods, can be characterised. RNA profiling has the additional advantage

over other alternatives that it can be co-extracted with DNA from the same sample, limiting stain consumption. In the immediate future, work is required to continue to develop interpretation strategies to combat signal imbalances, marker dropout and drop-in and stochastic amplification effects. In addition, further work is needed to agree interpretation thresholds, with a balance to be struck between relatively high detection thresholds to limit false positive marker callings, at the potential cost of missing some cell types present in a complex mixture. Whilst most interpretation methods use a binary 'present/absent' call, more recent research has focussed on a probabilistic Bayesian approach, and this appears to be an avenue for further work. There remain issues with some markers shared between body fluids, particularly problematic in mixed body fluid stains. Consequently further research is required to identify more characterising markers. Studies have also identified that the level of gene expression of some mRNAs is not well correlated with the prediction of DNA yield, indicating research opportunities to provide guidance on how mRNA profiling and DNA analysis can be better utilised together.

The shorter molecules of miRNA offers further potential where samples are degraded, with far greater sensitivity than mRNA methods, and potentially offer greater discriminating power between body fluids; therefore in the future this may offer more opportunity to discriminate in mixed body fluid stains. However, the methodology and interpretation is some years behind mRNA, and there is much work to be done in characterising distinguishable body fluid markers. The number of body fluid-specific miRNAs is currently limited utilising current microarray-based techniques, but the future application of massively parallel sequencing (MPS) has the potential to screen for miRNA markers at a genome-wide level.

DNA methylation, being DNA-based, does have the advantage over tissue-specific mRNA typing that it connects directly to the identification of the individual by DNA analysis, as well as benefitting from the greater stability of DNA. It also can be used after DNA testing, without a decision made pre-analysis—unlike mRNA/miRNA which requires co-extraction. This is particularly helpful post-DNA analysis if multiple donors are identified. There are however significant challenges that future research needs to address if the method is to have practical application. Most body fluids comprise multiple cell types, each expressing different distinct methylation patterns, so there is a need to define further robust markers, particularly to distinguish between saliva and vaginal secretions which are currently proving challenging. As the authors identify, multiplex MPS-based methods may offer potential but much method development is needed. Current analysis requires large quantities of high-quality DNA and can degrade much of the DNA present, whereas low DNA levels, such as may be encountered in casework, can produce stochastic variations. Future method development needs to look at more realistic casework-type samples, including mixed stains where methylation may need to be allied with other methods such as mRNA analysis.

On the basis of the current technology, metagenomic analysis appears to have to make the longest journey to be useable in a meaningful manner to forensic science. The authors identify several major hurdles, not least of which is that the microbial markers generally seem to identify the body site of the body fluid, not the identity of the fluid. In addition, issues remain around weak signals (false positives) from

similar microbial populations elsewhere on the body, or from non-human sources such as animal samples or foodstuffs, and there remain issues around DNA extraction and PCR amplification preferentially skewing the apparent populations. Significant further research is required to establish if microbial populations may be more confidently associated with specific body fluids, to investigate the extent of person-to-person variation which has been reported by some research and to consider how, if implemented in casework, inadvertent bacterial contamination by the operator or from the crime scene/laboratory environment could be controlled.

Perhaps the most exciting emerging technology for the forensic practitioner seeking to identify body fluids is the use of biosensor methods. The key advantage of biosensor technology, that distinguishes it from many of the other methods considered earlier, is that the procedure is utilised at the *searching* phase, rather than confirming the identity of stains that have already been found. Future method development needs to look at how the method can evolve from one of measuring fluorescence in a solution via spectrofluorometry to one of measuring fluorescence (or other indicator) directly from treated garments or other substrates, including outside locations where conditions may not be able to be darkened artificially. The non-quantitative nature of this method may be an issue in some circumstances and so research needs to address if the technique response can distinguish between, for example, differing volumes of neat and dilute body fluid dispersed over equivalent surface areas of a substrate. Studies have identified candidate protein biomarkers in several body fluids of forensic interest, however, with a few exceptions, the antigen profiles of many of these are unknown and need far more work to characterise, which would allow the development of both biosensors and more conventional existing technology multiplex assay systems.

In conclusion, the challenge for any future body testing methodology is primarily one of overcoming the current sequential linear process of testing, for example using a multiplex approach that tests simultaneously for multiple body fluids (including body fluids for which no conventional tests currently exist). Of particular benefit is if the test protocol can be allied to identifying the donor via DNA analysis in the same test regime. Whilst many of the approaches discussed offer ways of confirming the body fluids within a stain already identified on an item, of even greater value to the forensic practitioner are those methodologies that can be utilised as searching tools, suitable for screening clothing at the laboratory bench, or upholstery, carpets and other surfaces at crime scenes.

A common theme emerges during the review of the emerging technologies—namely the need to make the transition from developmental methods that can identify neat, unadulterated body fluids in perfect laboratory conditions, to identifying casework samples of sub-optimal degraded, aged, mixed body fluid stains on assorted substrates. The research to this point has identified possible methods that offer great potential, and some methods have already evolved to a limited introduction in casework. However, extensive method development and validation studies are needed to define further the accuracy, reliability and limits of detection of the techniques discussed in the review. The very specific requirements of the forensic arena mean that future methodologies must also dovetail with many other diverse search methods

and evidence recovery methods in use at a modern forensic science laboratory or at the crime scene. It is therefore essential that during future method development and validation studies, those in academia and industry engage with the end-user forensic practitioner, so that the developed methods can best meet the needs of the practitioner, the forensic science providers, and the wider Criminal Justice System.

Chapter 7
Investigating the Age of Blood Traces: How Close Are We to Finding the Holy Grail of Forensic Science?

Maurice Aalders and Leah Wilk

Abstract Blood traces found at crime scenes often comprise pivotal information regarding the events and individuals associated with the crime. Nowadays, even minute amounts of blood allow retrieval of a whole host of such 'profiling' information: e.g. diet, life style, age, gender. However, establishing any forensic value of such traces necessitates a veritable connection to a crime. The age of a blood trace, i.e. the time of its deposition, is crucial in this effort. This far-reaching forensic implication as well as the lack of currently validated and accepted trace dating methods, render blood stain age estimation the *holy grail* of forensic science. In its pursuit, several methods which determine the time since deposition of blood traces by probing different aspects of the trace degradation process have been proposed and explored. The present chapter collates and discusses current research investigating some of these blood trace ageing methods and their practical application in three categories. The first category comprises techniques which require trace sampling and consume these samples in their entirety during the analysis process. Similarly, the techniques in the second category require sampling of the blood trace but leave the sample intact for further analysis. Lastly, the third group of methods requires neither sampling nor contact. This, in turn, allows in situ analysis of the trace in question. The following operational aspects pertaining to these three categories are discussed in more detail: (i) required sample preparation, (ii) practical implementation and (iii) necessary operational skills. These aspects largely determine the suitability for forensic practice. Technology maturity (i.e. practical applicability) is quantified using the Technology Readiness Levels (TRL) as defined by the NASA/Airspace systems.

7.1 Introduction

Blood traces found at crime scenes can contain a wealth of information regarding the events and individuals associated with the crime. Recent studies have significantly

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diversified the accessible types of this 'profiling' information, retrievable from even minute amounts of blood: e.g. diet, life style, age, gender. Prior to such analysis, the blood traces must be localised, identified and chemically analysed, preferably directly at the crime scene, to aid the selection of relevant traces for further laboratory based processing. Accordingly, detection and identification of blood traces represent the first challenges to blood trace-based donor profiling. Moreover, depending on their underlying principle, the methods employed to this end can have implications for ensuing trace analyses.

Currently, the detection and identification of biological evidence both rely on visual inspection by the crime scene investigators (CSI), sometimes with the aid of forensic lights (coloured light sources) to enhance or create contrast between a presumed trace and its substrate. Once a trace is found, it is subjected to one or more presumptive tests for specific biological fluids (e.g. blood, semen, saliva). The most common screening test for blood utilizes a catalytic reaction inducing chemiluminescence (faint glow) or a colour change in the presence of blood as described in the previous chapter. Recently, immunological tests capable of identifying human blood-specific proteins have also become available. These immunological tests, however, require sampling and consume material. Following a positive presumptive test at the crime scene, the trace is sampled for a confirmatory test at the forensic laboratory. This investigation protocol is labour-intensive and carries the double risk of sampling redundant or irrelevant traces while missing relevant traces altogether. Furthermore, it isolates the sampled traces from their respective contextual information.

Blood traces sampled, processed and analysed in this way may contain crucial information regarding the crime; however, in order for a trace to have any forensic value, the determination of a veritable connection to a crime is imperative. The time of deposition, calculated from the 'age of the bloodstain', is an essential parameter in this effort. Furthermore, if bloodstains are clearly crime-related, their age may reveal the time of the crime or even the sequence of events. Owing to this high forensic value and the lack of currently validated and accepted trace dating methods, blood stain age estimation is still considered the *holy grail* of forensic science.

In its pursuit, many techniques have been proposed which probe the blood degradation process in order to determine the age of blood traces. Ideally, such methods should measure a (preferably) donor-independent and predictive (i.e. time-dependent) quantity in situ with known responses to environmental factors such as temperature and humidity. Moreover, the embedding of such a technique within the routine crime scene processing work-flow necessitates compliance with the following field-specific requirements: equipment portability, sample preservation and the operational skills of the CSI. When in situ trace analysis is not possible, techniques which require trace sampling, e.g. for measurements under controlled conditions, but preserve the sample for further processing can be employed. Lastly, destructive methods can be used which not only require trace sampling, but also consume the sample. While this is the least preferable approach, the high forensic value of blood trace ages would usually outweigh the disadvantages of such destructive techniques.

A survey of available literature reveals that many methods lack practical implementation, despite their reported success in the laboratory. The successful translation

from lab technology to field application is not only determined by the aforementioned operational constraints, the robustness of the technique and admissibility in court but also by the possibilities for commercialization.

This chapter aims to provide an overview of current research investigating blood trace ageing methods and their practical application. The research discussed here will be categorized into three main groups: the first category requires sampling and consumes the sample in its entirety during the chemical processing; the second group of techniques requires sampling of the blood trace but preserves the sample for further analysis; the third group requires neither sampling nor contact and therefore completely preserves the trace in its original context allowing in situ analysis of the trace in question. Within these three categories the following aspects will be discussed for each group of techniques: (i) necessary sample preparation, (ii) practical implementation and (iii) required operational skills. These aspects largely determine the acceptance/applicability in forensic practice. Technology maturity (i.e. practical applicability) will be quantified using the Technology Readiness Levels (TRL) as defined by the NASA/Airspace systems. The scale is depicted in detail in Fig. 7.1 and comprises 9 levels, where TRL 9 denotes a fully validated and launched method.

7.2 Lab-Based Techniques

7.2.1 Invasive and Sample Consuming

7.2.1.1 DNA/RNA Degradation

DNA extracted from biological stains can be used to identify the trace donor; this information is commonly used to establish links between the perpetrator, the victim and the scene of a crime. Additionally, the ex vivo degradation of DNA/RNA can be used as a measure of the trace age. The feasibility of this approach has been explored by several research groups. In 2005, Anderson et al. used real-time reverse transcriptase PCR to show that, over the course of 150 days, the ratio of different types of RNA (mRNA versus rRNA) exhibits a linear relationship with trace age [1, 2]. To this end, 10 µl bloodstains were dissolved and consumed in the process, and the authors showed that the possibility to simultaneously extract DNA and RNA is beneficial for further processing. The influence of environmental factors as well as donor gender, age and ethnicity had yet to be studied. In a follow-up study 6 years later, the same group utilized this technique in conjunction with multivariate analysis of the trace age-related ratio of different RNA segments [3]. This approach allowed robust distinction between three blood trace age categories: (i) fresh; (ii) 6 days; (iii) 30 days or more. Some of the 30-day-old samples could be distinguished from 90-day-old samples, while others could not. More recently, in 2012, Simard et al. demonstrated the feasibility of RNA marker degradation-based blood stain age estimation [4]. Fur-

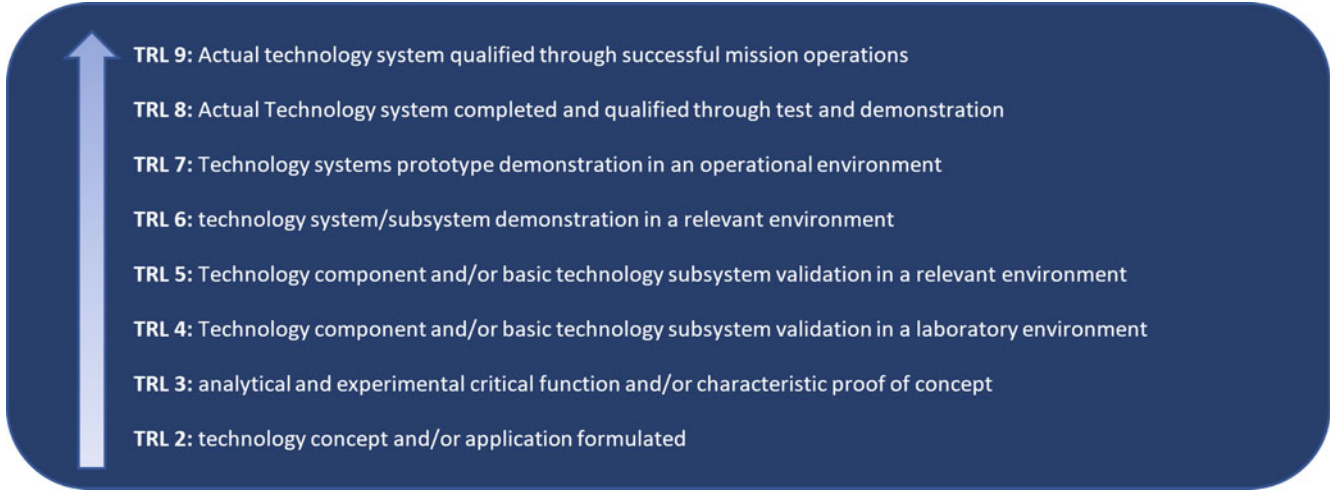

Fig. 7.1 Technology readiness levels (TRL) as defined by the NASA/Airspace systems. This scale is used throughout the chapter to classify the practical applicability of the discussed methods

thermore, the authors found ratios to be ineffective age measures due to the similar decay kinetics of the various markers, rendering such ratios time-independent. This study further showed that sample storage at −80 °C prevented sample degradation. The effects of other storage and environmental conditions were not studied. In 2013, donor gender was found to have a significant influence on the method proposed by Anderson et al., necessitating further research [5]. More recently, in 2017, Alshehhi et al. studied the degradation of three mRNA markers (HBA, PBGD, HBB) and two microRNA markers (miR16, miR451), along with three reference genes (18S rRNA, ACTB mRNA, U6 snRNA) as a blood trace age measure using blood samples from 10 donors [6]. While potential feasibility was suggested, no follow up has been published to date. Finally, Mohammed et al. investigated mRNA levels as a measure for bloodstain ages up to 55 days [7]. More specifically, Caspase-1 and Caspase-3 showed a tendency to decrease over time. All studies discussed here remained within the lab validation phase and revealed ex vivo RNA degradation to be less well understood than its in vivo counterpart. At the time of editing this chapter, no further lab or field validation studies were available, resulting in a TRL score of 3 or 4 for DNA/RNA-based blood stain age estimation. However, potential future introduction of these methods in forensic practice would be facilitated by the existing DNA/RNA facilities and experts in forensic laboratories.

7.2.1.2 Haemoglobin Degradation

Electron paramagnetic resonance (EPR), also called electron spin resonance, studies chemical species (especially metal complexes and organic radicals) with unpaired electrons. Contrary to the more common Nuclear Magnetic Resonance (NMR) spectroscopy, which measures nuclear spin, EPR probes electron spin. EPR is typically performed by placing the sample in a magnetic field, the strength of which is varied, while microwaves of a fixed frequency in the GHz range serve as probe. The studied effects within EPR samples are very sensitive to local environments and, because of the short relaxation times, often necessitate very low measurement temperatures [8]. Miki et al. and Sakurai et al., both assessed the feasibility of EPR-based blood trace dating, following the hypothesis that the spin configuration of the iron atom within the haemoglobin molecule will change over time [9, 10]. They found that the logarithm of the ratio of two specific spin states and the logarithm of sample age exhibited a linear correlation for up to 432 days with an error range within 25%. While this method has to be performed under controlled laboratory conditions, advantageously, the blood trace can be dry sampled, i.e. ‘scraped off the surface’, and transported to the laboratory. The researchers stated that environmental factors such as differences in light exposure and fluctuations of storage temperature did have an influence on the changes of the EPR-active compounds, but these factors were not further investigated. EPR devices are not standard inventory at forensic laboratories and the measurements require highly specialised personnel. Together, these conclusions and the lack of continued method development classifies the technique at TRL 4.

7.2.1.3 Biomolecule Degradation

In 1997, Andrasko investigated the feasibility of using High-Performance Liquid Chromatography (HPLC) for age estimation of bloodstains deposited on clothing [11]. HPLC is a method of separating, identifying and quantifying each chemical component in complex mixtures. To this end, the sample is injected into a flowing solvent and both will be "pushed through" the stationary phase of a separation column. Separation is achieved by specific chemical or physical interactions of the sample with the stationary phase: different chemical components in the sample travel through the column at different velocities and hence exit the column at different times, at which point they will be detected using various means e.g. using their light scattering, absorption or fluorescent properties. Already in 1992, the technique was explored by Inoue et al., who found an 'unidentified decomposition peak', potentially useful for ageing stains [12]. Andrasko studied several other ageing processes and found some suitable for the estimation of the age of blood stains [11]. Notably, and of considerable practical importance, the ratio of the different peaks formed by the ageing process is practically independent of environmental temperatures between 0 and 37 °C. Despite these promising results, the associated costs and required operational skill levels may hamper integration of HPLC into routine forensic practice. With a current TRL of 4, additional research is necessary to advance this technique towards practical implementation.

7.2.1.4 Amino Acid Conversion

Another interesting approach is based on the aspartic acid racemization (AAR) rate in ageing bloodstains. AAR is a common process during the natural ageing of proteins and therefore used in medical applications to assess protein ageing. Although this destructive method is established for the dating of other (slow turnover) tissues, such as tooth tissue and fossils, only one author [13] applied the technique to aging bloodstains. The procedure involves dissolving the present amino acids, followed by a centrifugation step and subsequent gas chromatography (GC) analysis. Initial experiments at various temperatures were successful and revealed a linear relationship between D-aspartic acid (D-Asx) content and age. However, the relationship was also found to be highly sensitive to the environmental temperature. As only a few stains were measured and no follow-up research has been published to date, this approach currently remains at TRL 3.

7.2.1.5 Metabolomics

All methods described in this chapter aim to determine the absolute time of deposition. The temporal resolution can be increased further by determining both the moment of deposition as well as the time of deposition within the circadian (24 h) night/day cycle. The idea to use circadian biomarkers present in the blood was

explored by Kayser et al. in 2010 [14]. Two circadian hormones were found suitable for trace deposition time estimation and in 2016 the same group demonstrated that messenger RNA (mRNA) biomarkers significantly improve the prediction accuracy [15]. This idea was extended in 2018 when the suitability of metabolites, measured using a targeted metabolomics approach, were explored as potential measure for trace age. An integrated approach, using time prediction modelling in conjunction with a combination of these metabolites and the hormone and mRNA biomarkers identified in the previous studies, achieved an improved prediction accuracy reaching AUCs of 0.85, 0.89 and 0.96 respectively [16]. The authors believe that this approach currently is at TRL 3.

Advantages and Limitations Most of the proposed blood trace dating approaches discussed in this section employ analytical chemistry methods. Accordingly, the necessary equipment is generally located in a chemical laboratory, usually expensive and must be operated by specialized personnel. The required sampling and processing steps entail the following risks: trace contamination, material loss, and change of the chemical composition of the sample. Operational risks and practical constraints notwithstanding, such lab-based devices usually outperform most portable techniques in terms of stability, reliability and data quality. Moreover, the required equipment is usually part of the standard inventory in a forensic lab. Lastly, some of these laboratory techniques are slowly progressing towards mobile applications, however, usually at the cost of robustness and sensitivity.

7.2.2 Invasive but Sample Preserving Techniques

7.2.2.1 Exploiting Biomolecule Degradation

Raman spectroscopy is an optical technique based on inelastic (so-called Raman) scattering of light, a process in which photons lose or gain energy upon interactions with vibrating molecules. This, in turn, provides chemical 'fingerprints' of cells, tissues or biological fluids and yields detailed information about the chemical structure of the present (bio)molecules. Consequently, Raman spectroscopy has been explored extensively in the medical field and, more recently, also for forensic applications. To measure a Raman spectrum, the sample has to be illuminated with a light source providing a stable output, usually a diode laser. The optimal wavelength depends on the material under investigation and is a trade-off between strong Raman scattering signals at short (UV-Vis) wavelengths at the cost of more unspecific background (fluorescence) signal, or less disturbance from the background signal at longer wavelengths at the cost of lower Raman signals. The four most widely used wavelengths are 405, 532, 785 and 1064 nm. Recording such Raman signals requires a sensitive spectrograph, which makes up most of the relatively high costs of Raman systems. Many application papers show measurements of pure components on a non-fluorescent, and non-Raman scattering substrate such as aluminium foil. Translational investigations

therefore often rely on sampling and purification. For blood trace age estimation, blood was mostly deposited on aluminium foil-covered microscope slides.

In 2016 and 2017, the Lednev group demonstrated the feasibility of using Raman spectroscopy for blood stain age estimation over a time range of 200 days [17, 18]. Time since deposition predictions were performed employing two statistical regression algorithms: partial least squares regression (PLSR) and principal component regression (PCR). Error analysis revealed an overall accuracy of ~70% for the models to correctly predict the time since deposition at each time point. Besides trace dating, the technique also enables discrimination of human and non-human blood traces [19]. Taken together, these findings indicate that translation of this approach from the lab to forensic practice still requires major technological developments, rendering Raman-based blood stain age estimation TRL 5.

7.2.2.2 Exploiting Structural Conversion (Sample Elasticity)

Atomic force microscopy (AFM) is a technique capable of providing elasticity measurements of red blood cells. This, in turn, can be used to monitor time-related changes in the elasticity of red blood cells in ageing blood traces. Unlike Raman spectroscopy, AFM measurements are not sensitive to substrate interference; however, since AFM measures minute displacements, it is extremely sensitive to vibrations. As a result, AFM measurements have to be performed in a vibration-free environment rendering the development of portable systems challenging. While recent developments show a trend towards increased ease of use and portability, a high level of operational expertise is still required for data acquisition and interpretation. AFM-based bloodstain age estimation was explored by Strasser et al. [20] and Smijs et al. [21] with results indicating potential feasibility. Both groups reported an age-related decrease in red blood cell elasticity, caused by drying and coagulation processes. Dating of bloodstains was possible for up to 8 days with a reasonable accuracy at days 6 and 7. Despite these promising results, many possibly influential factors remain to be investigated before moving to the field validation phase, rendering this approach TRL 4.

Advantages and Limitations Similar to the previous section, the techniques discussed in this section require sampling. However, advantageously, they do not consume or alter the sample allowing subsequent complimentary analyses. Reasons for sampling and transportation of the sample include substrate interference or the necessity to dissolve the blood for the measurement. While the sample preservation allows ensuing additional analyses, the sampling requirement introduces trace contamination risks. Furthermore, sampling may cause a change in chemical properties such as the relative amounts of haemoglobin derivatives. This problem is frequently overlooked, as liquid blood or even dissolved blood samples represent the analytical starting point in some publications, entirely negating any adverse sampling effects. These limitations notwithstanding, some of the techniques presented in this section are potentially portable and could be used in field work, e.g. Raman spectroscopy.

7.3 Portable and Non-invasive Techniques

This section describes the most recent technological developments aimed at providing blood trace age estimates in an entirely non-contact fashion, i.e. in situ and without the need to sample. Such methods are extremely sought after as they preserve the original trace context; prevent trace contamination and destruction, while potentially reducing the required operational skill level and workload by the same token.

Experimentally, all approaches discussed here utilize visible and near-infrared light to probe and monitor the chemical composition of ageing blood traces. The employed data acquisition tools vary broadly in sophistication: from ubiquitous smartphone cameras to portable hyperspectral imaging systems and lab-grade spectrometers. Similarly, data processing strategies range from statistical classification techniques to advanced physical models describing the light-sample interaction. Consequently, some of the associated advantages and limitations are strategy-specific and need to be discussed and weighed separately. In general, non-contact methods employing visible light all probe the degradation state of one of the main constituents of blood, the oxygen-transporting protein haemoglobin. The degradation of this protein is associated with a visible change in blood stain colour (Fig. 7.2), from bright red to dark brown.

Upon contact with air, haemoglobin immediately binds oxygen molecules; this fully oxygenated form of haemoglobin is referred to as oxyhaemoglobin (HbO_2) and is responsible for the bright red colour of fresh blood. Due to the continued exposure to air, HbO_2 subsequently converts (auto-oxidizes) to a molecule known as methaemoglobin (metHb). As a result, the colour of the blood stain changes to a darker, brownish red. Finally, the visible ageing process of haemoglobin is completed by the degradation (denaturation) of metHb to hemichrome (HC), resulting in a dark brown appearance of the blood stain. Techniques which are capable of objectively quantifying such colour changes, i.e. spectroscopic techniques, are therefore, in theory, ideally suited to probing this ageing process.

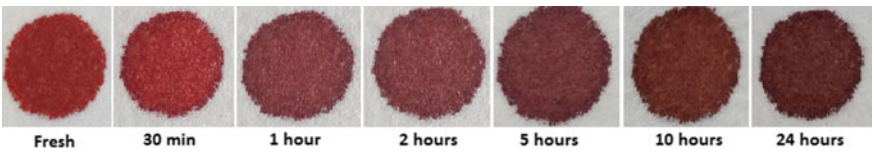

Fig. 7.2 Colour change observed in ageing blood stains

7.3.1 Low-Cost Approaches

7.3.1.1 Smartphones

Some of the most recent investigations into optical blood stain age estimation are geared towards the use of smartphones for both data collection and analysis. If successful, such techniques would be conceivably cost-efficient by lowering the required operational expertise, workload and equipment cost. Consequently, these approaches are particularly attractive in low resource settings.

Recently, two studies explored the feasibility of smartphone-based age estimation of blood stains. The first one [22] utilized digital colour images recorded with a smartphone camera in conjunction with a statistical classification (machine learning) algorithm. All pixels of the recorded digital images comprise information about *three* primary colours: red (R), green (G) and blue (B). These values can be used to monitor the expected change in blood stain colour over time. In this study, however, the recorded RGB values were first transformed to intensities in a complimentary colour model, the four colour (CMYK) model as these exhibited a stronger correlation with blood stain age. The CMYK model is primarily used in the printing industry and represents all visible colours as a combination of *four* primary colours (cyan, magenta, yellow and black) instead of the previously mentioned three. Following the transformation, a so-called Random Forest machine learning algorithm was applied to estimate the age of the blood stains. This approach allowed 100% accurate age estimation for blood stains deposited on filter paper for several time periods between 1 h and 42 days under controlled environmental conditions. For blood stains deposited on six light-coloured common household substrates, the correct classification rate dropped to 83%. In this case, the age estimation task was limited to three categories: younger than one day; between one day and one week old; older than one week. Interestingly, in both cases, the classification algorithm performed best for the youngest and the oldest stains.

The approach pursued in the second, and more recent, of the two studies also employed a combination of digital images recorded using a smartphone and a colour value transformation [23]. Here, RGB values were mapped to brightness values by assigning every RGB triplet the minimum of its three values. This colour value transformation seems to reduce the sensitivity to the visible ageing process, as changes in the brightness value were limited to ages between 0 and 42h.

Advantages and Limitations Clear advantages of smartphone-based methods are that, in principle, they are fast, simple and cheap and require minimal training of the end user. Despite some promising results, however, considerable challenges remain. The perhaps most obvious limitation is that of substrate colour. Dark and otherwise coloured substrates mask age-related variation in the measured colour values, eroding any age estimation potential. Moreover, the experimental set-up of the first study included a purpose-built white foam box containing the sample to be imaged. Imaging of the blood stains was carried out through a small hole at top of the box, presumably to achieve homogenous illumination conditions. This requirement may hamper the

application of this method in forensic field work. Furthermore, the used statistical classification model is device specific and therefore has to be rebuilt specifically for every smartphone. Lastly, the conversion to CMYK values seems to introduce a sensitivity to sample light exposure, as the magenta values measured on blood stains exposed to natural sunlight differed significantly from those of blood stains of the same age not exposed to sunlight. Together, these aspects classify smartphone-based blood stain age estimation as TRL 4.

7.3.2 Portable Lab-Grade Equipment

7.3.2.1 Reflectance Spectroscopy

As mentioned earlier, the colour change observed in ageing blood stains is associated with the successive formation of three haemoglobin derivatives: HbO_2, metHb and HC. Figure 7.3 shows the light absorbing properties of these three compounds as a function of wavelength, i.e. light colour, in the range of 500 nm (green light) to 800 nm (near-infrared light). Light absorption and reflection are natural inverses of each other as increased absorption at a certain wavelength decreases the amount of light available for reflection. There are pronounced differences in these three wavelength-dependent absorption profiles and hence also in the colour of the three haemoglobin derivatives. Furthermore, this graph clearly shows a variety of compound-specific spectral features such as highly localised absorption peaks. Consequently, employing measurement equipment of sufficient wavelength samples and resolution allows more specific monitoring of the blood stain ageing process.

A technique ideally suited to this purpose is reflection spectroscopy as it records the light reflection profile of a sample as a function of wavelength. Accordingly, there

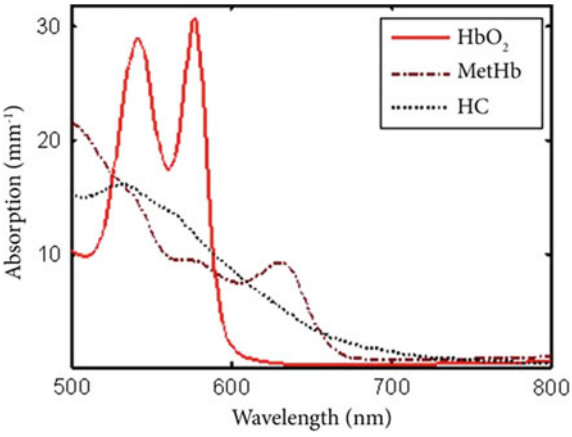

Fig. 7.3 Light absorbing properties of three haemoglobin derivatives formed in ageing blood stains. Reproduced from: G. J. Edelman, "Title: Spectral analysis of blood stains at the crime scene" Ph.D. thesis Faculty of Medicine (AMC-UvA), http://hdl.handle.net/11245/1.416827

have been a number of studies investigating the potential of reflection spectroscopy for blood stain age estimation.

The most recent study investigated the use of visible reflectance spectroscopy in conjunction with chemometric data analysis strategies [24]. Here, three different statistical classification algorithms were benchmarked against each other to determine the most effective age estimation method. Blood stains were deposited on glass slides and reflection spectra were recorded between 500 and 780 nm at ages ranging from 2 h to 45 days. The study found that the most effective age estimation strategy consisted of first reducing the dimensionality (number of data points) of the reflection spectra using partial least squares regression and using this transformed data to build age estimation regression models by means of least-squares support vector machines. This approach achieved a root mean square error of prediction (RMSPE) of 42.792h. Another study investigated the effectiveness of a slightly different strategy for dimensionality reduction and classification, employing Fourier transformation as feature selection tool followed by Linear Discriminant Analysis for the classification task [25]. Using this approach correct classification (±1 day) of blood stains deposited on a white glazed tile and younger than 19 days was possible for 83.3% of all tested blood stains.

As mentioned above, reflectance spectrometers, of sufficient wavelength samples and resolution, should allow the monitoring of compound-specific light absorption features. However, due to relatively complex light-sample interactions (scattering), the measured blood stain reflectance will usually depend on these absorption profiles in a non-linear way (Fig. 7.4). Consequently, reflectance-based quantification of haemoglobin derivatives necessitates a physical model accounting for these light-sample interactions. The use of such models for the purpose of blood stain age estimation has been the subject of several studies in the past. Most notably, one of these methods recently matured to being applied and validated at crime scenes [26]. In this approach, the complex light-sample interactions (including substrate reflectance) are accounted for by employing a so-called light transport model to

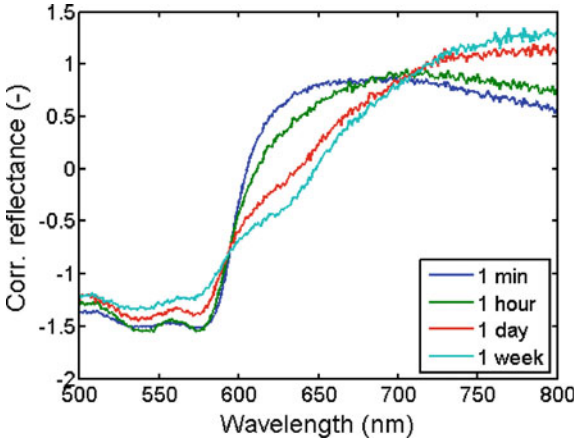

Fig. 7.4 Reflectance spectra of blood stains on cotton of different ages exhibiting absorption features of the different haemoglobin derivatives. Reproduced from: G. J. Edelman, "Title: Spectral analysis of blood stains at the crime scene" Ph.D. thesis Faculty of Medicine (AMC-UvA), http://hdl.handle.net/11245/1.416827

describe the blood stain reflectance. This model is derived by describing blood stains as two-layer systems (blood atop a substrate) and solving one of the fundamental physical equations describing light propagation. By applying known physical constraints (layer thickness etc.), a theoretical prediction of the wavelength-dependent blood stain reflectance can be derived. This, in turn, allows estimation of the contribution of each of the three haemoglobin derivatives by comparing measured and theoretical spectra and determining the best fit. Finally, these estimated contributions yield fractions of the present haemoglobin derivatives. Figure 7.5 shows the change of these fractions as a function of blood stain age, in particular the decrease of the HbO_2 over time, rendering it a suitable measure of blood stain age. In order to use the HbO_2 fraction of an *unknown* blood stain to predict its age, a database was created consisting of HbO_2 fractions of multiple blood stains on cotton measured under laboratory conditions for a period of 200 days. Utilising this data to build a statistical regression model then allows the calculation of a predicted age along with a 95% confidence interval. This method was then tested at a real crime scene where it enabled differentiation of two groups of blood stains likely deposited at different times. Another possible approach compares the three haemoglobin fractions of an unknown stain to a similar database containing all three fractions for different blood stain ages [27]. Finding the smallest difference between the three measured fractions and any database entry then yields a predicted blood stain age with a median relative error of 13.4%. Both methods allow non-contact age estimation for up to 200 day old blood stains.

The reaction kinetics of haemoglobin degradation strongly depend on environmental factors such as temperature and humidity. Specifically, the reaction rate of the entire degradation process increases with temperature, while increased humidity levels decrease the rate of the metHb to HC conversion. As a result, employing either databases or training sets containing age-specific data acquired under a particular set of environmental conditions would lead to incorrect age predictions, if

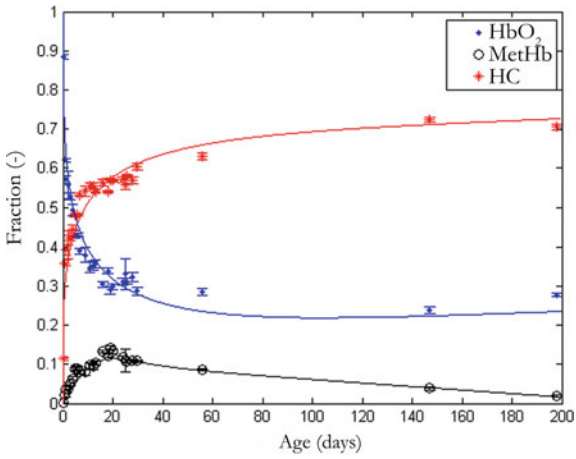

Fig. 7.5 Estimated fractions of the three haemoglobin derivatives as a function of time (reprinted from reference [27] with permission from Elsevier)

used to estimate the age of a blood stain aged under different or unknown conditions. In forensic practice, however, it is often the *relative* age of blood stains which is of interest. Therefore, if environmental conditions are unknown but conceivably identical for two sets of blood stains, comparison of their estimated haemoglobin fractions will still reveal valuable information: significantly different fractions likely indicate different times of deposition [27]. This comparison necessitates estimation of the haemoglobin fractions and hence the use of the light transport model discussed above.

Advantages and Limitations The use of reflectance spectroscopy for the non-contact age estimation of blood stains is associated with several advantages over the low-cost approaches discussed earlier. Firstly, it enables more specific monitoring of the ageing process (haemoglobin degradation) due to the larger number of recorded wavelength (colour) samples and generally increased wavelength resolution; this, in turn, increases the statistical strength of the results obtained in this way. Secondly, using suitable fibre-based probes inherently ensures homogenous sample illumination. Thirdly, to date no sunlight exposure sensitivity has been observed in data recorded using reflectance spectroscopy. Aside from these advantages, and just as in smartphone-based approaches, the use of classification algorithms requires no a priori knowledge about reaction kinetics or advanced physical modelling skills. However, when using reflection spectroscopy, the increased number of recorded data points significantly diversifies data (pre-)processing options, such as data dimensionality reduction. While smartphone-based measurements are perhaps the most intuitive to carry out, spectroscopic measurements are also very simple operations requiring minimal user training. Bedsides its many advantages there are also some limitations to the use of reflection spectroscopic methods for the non-contact age estimation of blood stains. Dark substrates strongly reduce the measured reflectance thereby diminishing the information content of the reflectance spectra. Consequently, no meaningful results can be obtained from visible reflectance spectra measured on dark backgrounds. Coloured substrates pose a problem only in combination with statistical classification methods. Here, libraries would have to be built containing example spectra of blood of different ages on every encounterable substrate. Lastly, hardware requirements render reflectance spectroscopy a more expensive approach than smartphone-based methods. It is important to note that, the use of the light-transport model mentioned above is not hampered by coloured substrates as it explicitly accounts for the substrate influence on the measured reflectance spectra. Moreover, this approach is the only non-contact age estimation method tested in forensic field work to date. Consequently, this approach is the most advanced of all blood trace age estimation techniques at TRL 9.

7.3.2.2 Hyperspectral Imaging

In digital photography, rendering of all perceivable colours is achieved by recording information about three primary colours for every pixel in three separate channels:

red (R), green (G) and blue (B). These types of images are therefore most commonly referred to as RGB images. As discussed earlier in the low-cost approaches section, these three channels do not sufficiently capture the colour changes observed in ageing blood stains. This shortcoming can be addressed by increasing the number of wavelength samples, i.e. colour channels, for instance using a hyperspectral camera. Such cameras successively record reflectance images at numerous wavelengths (colours) creating so-called hypercubes; every pixel of a hypercube therefore comprises an entire reflection spectrum (Fig. 7.6). Selecting regions of interest (ROIs) within the hypercube, e.g. a group of pixels constituting a blood stain, and averaging its spectra yields a reflection spectrum for the selected region, e.g. of the blood stain in question. Consequently, this enables non-contact age estimation of blood stains utilizing either the statistical classification methods or the light-transport model discussed in the reflectance spectroscopy section. The feasibility of such hyperspectral imaging approaches has been demonstrated in two studies in the past.

In the first of the two studies [27], blood was deposited on white cotton and left to age under laboratory conditions. Hypercubes, with a spectral range of 500–800 nm, were recorded of the sample at numerous time points between 0 h and 200 days. ROIs were then selected within these hypercubes to obtain blood stain reflectance spectra at every time point for further analysis. To this end, 8 ROIs comprising 25 pixels each were selected per hypercube. These 25 reflectance spectra were then averaged to yield 8 ROI-specific reflectance spectra per hypercube, i.e. per time point. Subsequently, three haemoglobin derivative fractions were calculated for every ROI and time point employing the light-transport model approach outlined above. Using these age-specific haemoglobin derivative fractions, a database was generated serving as a reference set in the age estimation for a comparable test set of blood stains. This test set was created by repeating the above ROI selection and haemoglobin fraction calculation steps for a set of neighbouring blood stains within the same

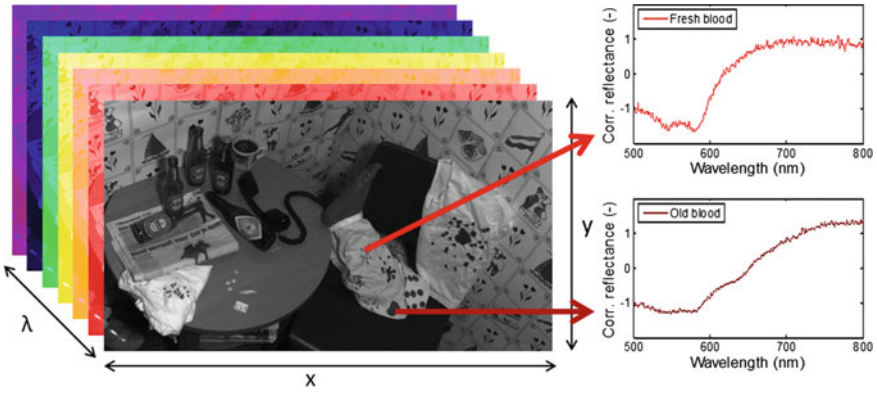

Fig. 7.6 Hypercube (left) of a mock crime scene and the reflectance spectra (right) of two pixels within two blood stains of different ages (reprinted from reference [27] with permission from Elsevier)

hypercubes. Non-contact age estimation was achieved by finding the reference set entry containing the most similar combination of haemoglobin fractions to the test set entry in question. This approach enables non-contact age estimation of blood stains for up 200 days with a median relative error of 13.4% of the true age. Unknown environmental conditions at the crime scene potentially render absolute age estimations inaccurate. In this case, relative ages, which are frequently of forensic interest, can still be determined. This information can also be derived from the haemoglobin derivative fractions using so-called clustering algorithms. These algorithms attempt to divide large data sets into smaller subgroups with shared properties. Here, similar combinations of the three haemoglobin derivatives indicate similar times of deposition. Consequently, blood stains grouped together on the basis of their haemoglobin derivative fractions are likely of similar ages. Figure 7.7 shows the results of this relative age estimation task overlaid on an image of the mock crime scene. All blood stains in the scene were grouped correctly, except for the youngest stains (orange ellipse) which were grouped with 2 day old blood stains while their true age was 0.1 days.

The second study investigated the feasibility non-contact age estimation of blood stains using hyperspectral imaging in conjunction with statistical classification methods [25]. To this end, hypercubes of two blood stains deposited on white printing paper were recorded daily for ages between 10 min and 30 days with a spectral range of 505–600 nm. Here, 10 ROIs of 25 pixels each were selected per blood stain yielding two databases containing 310 reflectance spectra each (10 for each age). One of the databases then served as training set for the classification algorithm while the

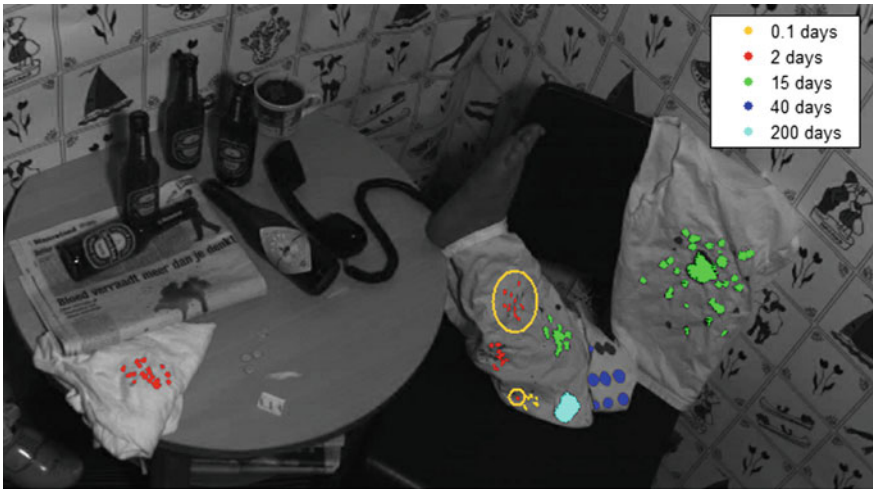

Fig. 7.7 Results of the largely automated relative age estimation task overlaid on an image of the mock crime scene. Almost all stains were grouped correctly, except for the stains in the orange ellipse which were included in the group of 2 day old stains while their true age was 0.1 days (reprinted from reference [27] with permission from Elsevier)

other one was used as a test set to monitor the accuracy of the age estimation. Allowing misclassification errors of one day, this approach achieves correct classification for 89.3% of the test data set. However, this rate significantly decreases for older ages, particularly for blood stains older than 14 days: only 53.5% of blood stains aged between 15 and 30 days are classified correctly.

Besides non-contact age estimation of blood stains another highly relevant forensic application of hyperspectral imaging is the visualization of latent blood traces on dark substrates. This additional capability is a by-product of the spectral range of these cameras, as they usually include the near-infrared part of the electromagnetic spectrum. While many dyes and colourants which strongly absorb in the visible do not absorb light in the near-infrared, some of the haemoglobin derivatives formed in the ageing process do. Consequently, contrast is created between blood traces and dark substrates in reflectance images recorded at near-infrared wavelengths (Fig. 7.8).

Advantages and Limitations Hyperspectral imaging not only records the trace context for later review, but also provides sufficient spectral (colour) information to accurately estimate the age of blood stains. Furthermore, hyperspectral imaging can be combined with standard image processing and computer vision tools, enabling highly objective and fast screening of entire crime scenes. This, in turn, significantly reduces the operational workload, while increasing the rate at which potentially investigation-guiding intelligence becomes available. Consequently, hyperspectral imaging, in conjunction with advanced signal processing, is certainly the most powerful emerging technology in the field of non-contact age estimation of blood stains. Until very recently, one aspect of the technique possibly hampering its adoption in forensic practice was hardware complexity, both physically and operationally. However, recent hardware developments have largely addressed and removed these

Fig. 7.8 Visualisation of latent blood traces on black cotton using hyperspectral imaging. Reflectance images recorded in the visible (left) and the near-infrared (right) part of the spectrum

shortcomings, providing forensic investigators with a highly portable and easy-to-use device requiring minimal to no additional training. Moreover, commercial software packages are being developed enabling forensic investigators to independently carry out non-invasive age estimation of blood stains at the crime scene. One remaining aspect potentially preventing the introduction of this technique in certain settings is the associated cost which exceeds that of smartphones and fibre-based reflection spectrometers. As a result, hyperspectral imaging for non-contact age estimation of blood stains can be classed at TRL 8/9.

7.4 Dark Substrates

7.4.1 Near-Infrared Spectroscopy

All techniques discussed in the previous sections probe and monitor colour changes observed in ageing blood stains. These colour changes, however, may be masked by dark substrates due to their strongly light absorbing properties. Consequently, approaches utilising visible reflectance are not applicable in cases involving such substrates. This limitation can be overcome by quantifying near-infrared reflectance instead, as many dyes and colourants which strongly absorb visible light do not absorb near-infrared light [28–30].

One study investigated the feasibility of non-destructive age estimation of blood stains on dark substrates using near-infrared spectroscopy. To this end, blood was deposited on black cotton and reflectance spectra were recorded between 1150 and 2500 nm. A statistical classification model was built and used to estimate the age of blood stains. This method achieves a relative RMSPE of 8.9% for blood stains on black cotton for ages between 0 and 30 days [28].

Advantages and Limitations If blood stains are found on dark substrates, near-infrared spectroscopy offers possibilities for age estimation where all methods utilising visible reflectance would fail. However, this approach is associated with some practical limitations. Firstly, the required equipment is not portable. As a result, samples have to be transported to a lab rendering it difficult or impossible to analyse blood traces deposited on immobile substrates. By the same token, necessary operational skill levels and associated costs exceed those of visible reflectance techniques. Taken together, these aspects render near-infrared spectroscopy for the age estimation of blood traces on dark substrates TRL 4.

7.5 Conclusions

This chapter showcases the most recent efforts in blood trace age estimation, a discipline frequently referred to as being in pursuit of the '*holy grail*' of forensic science. This designation reflects the unifying character of trace age information as well as its high forensic value by the same token: the age of a trace can establish a veritable connection between the trace and crime. Consequently, trace age effectively serves as a classification measure, reliably distinguishing relevant from irrelevant traces. So far, considerable strides have been made in pursuit of this '*holy grail*'. Numerous age-related processes along with their appropriate probes and potential quantification measures have been identified and investigated. In general, there are three classes of techniques: (i) those that require sampling and consume the material during the analytical process; (ii) those that require sampling but preserve the sample material for subsequent analyses; (iii) those that do not require any sampling and conserve the trace in situ. The associated practical advantages and limitations can be class- or even technique-specific. Accordingly, the most appropriate approach would have to be identified on a case-by-case basis. Overall, while many efforts seem to stagnate in a pre-translational phase, the methods currently within reach of the '*holy grail*', i.e. closest to being embedded in forensic routine- and field-work, utilise portable lab-grade equipment to objectively quantify subtle age-induced blood stain colour changes in situ. Most of these approaches, however, are limited to light-coloured substrates. In conclusion, while there are appreciable advances towards the '*holy grail*' of forensic science, its pursuit must be continued by addressing and overcoming the remaining challenges and hurdles.

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Chapter 8
End User Commentary on Investigating the Age of Blood Traces: How Close Are We to Finding the Holy Grail of Forensic Science?

Chris Gannicliffe

Despite significant efforts over the past decades, the forensic practitioner's quest to establish when a blood stain was deposited, and therefore whether it might relate to the offence, has remained elusive. In an age where DNA analysis continues to garner the attention, it is often overlooked that there are many instances where establishing *who* shed the blood stain is not necessarily the critical issue. For example, if a victim was known to the assailant, and particularly if there was a history of violence between the individuals concerned, then there is often a need to show that the blood matching the victim is not the result of some previous incident. It is not uncommon for the examination of a crime scene to find blood stains of assorted ages, the results of previous violent altercations between householders over the years, presenting the challenge of distinguishing between those blood stains which relate to the offence and those which are historic.

Whilst the techniques reviewed in this chapter clearly show that the biochemical characteristics of blood stains do indeed degrade with time, the challenge remains in identifying a specific blood marker that alters predictably in differing environmental conditions as the blood stain degrades. Moreover, having identified a suitable candidate, a technique must then be developed that can detect the marker with the necessary degree of precision and reproducibility to allow implementation into casework at both the crime scene and in the forensic laboratory. Currently, as the authors of the chapter have described, each of the methods show promise, but most require to evolve beyond what the authors describe as the 'pre-translational phase'. With the exception of reflectance spectroscopy and hyperspectral imaging, the authors classify most methods as having a Technology Readiness level (TRL) of just 4 or 5; that is, they remain at a relatively rudimentary validation stage, and have yet to make the transition to the real-world challenges encountered in casework. For example, most of the published studies discussed in the chapter have utilised idealised substrates and environmental conditions—understandably, as the studies sought to understand

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the inherent variations in the studied marker before introducing further variables. Many studies have utilised white cotton fabric as the substrate onto which the blood is deposited, but a robust blood ageing method needs to be able to accommodate a far wider range of substrates. It is known that the substrate has a significant influence on the size, shape and thickness of the resultant blood stain, which affects how quickly the blood stain dries (for example if the substrate is porous or non-porous), and which in turn may affect the characteristics of the marker in question. Blood stains at the crime scene and on clothing may be encountered on dark substrates where the stain is difficult to visualise, and many of the reflectance spectroscopy and spectrophotometric methods discussed in the chapter have yet to get to grips with the challenges this presents. The published studies discussed in the chapter have also been relatively conservative in the range of environmental factors considered, either not yet evaluating the effects of a full spectrum of some factors (for example only choosing three or four temperatures rather than a much broader range) or rarely considering others (for example humidity, or the effects of alcohol or illicit drugs which would be frequently encountered in crime scene blood stains). Even the environmental factor perhaps most widely studied, namely the effect of light on blood ageing, has rarely been evaluated under rigorous exposure regimes, and the details of precise wavelengths used are frequently undefined in publications.

For a forensic organisation working to ISO/IEC 17025 international quality standards, the validation process for a method to be implemented into casework requires a thorough, systematic assessment of all the factors that may influence the test result. In the case of a proposed body fluid testing method, the validation process typically considers body fluid samples from multiple donors, both male and female. A criticism of the published studies in blood ageing is that the research needs to consider a wider range of sample donors, considering inter-donor variation (for example, blood from differently aged donors), intra-donor variation (for example, hormone variation in females during the menstrual cycle), as well as the effects of medication, drugs and alcohol referred to earlier, and whether the technique may be adversely affected if it inadvertently encounters non-human blood or other body fluid stains, for example.

A typical validation process for implementation in casework also looks at the reliability of detecting the relevant marker when the body fluid stain is mixed with another material, for example another body fluid, or with cleaning fluid such as might be encountered where an offender has attempted to clean up a crime scene. It is not uncommon for blood to be mixed with another body fluid, for example with lung surfactant from a stab wound to the chest, with bile or vomit from internal bleeding, or with semen or vaginal fluid in a sexual offence. To date, it is probably fair to say that the methodologies are not sufficiently developed to have considered the effects on the test result of adulterating the blood stain with other material, but this is certainly an area one would expect to be addressed as a proposed method evolves.

Perhaps the most difficult challenge for any proposed candidate marker for blood stain ageing is to offer the chronological resolution necessary to make a meaningful, informative contribution to casework. For example, in some instances (albeit rare ones) it may be necessary to distinguish between blood shed a few hours earlier and blood shed days before. In other cases the question may be whether the blood was

deposited a few weeks ago or several months ago. Crime scenes may not be examined for days after the offence when the blood was shed, or the clothing of the suspect may not be examined in the laboratory for many weeks. As a consequence, if the competing scenarios are whether the blood stain was the result of the offence or an unrelated incident five days earlier, then if the suspect's clothing is not examined at the laboratory until four weeks later then the technique in reality has to distinguish between blood stains thirty days old or thirty five days old. The older a case is, the greater this problem will be—consider a cold case investigation where the blood stains might be thirty years old yet the required resolution to distinguish between scenarios might be a matter of days!

A single technique may not ever be able to offer these different levels of resolution, and it might be unrealistic to expect some especially challenging issues such as the cold case example to ever be resolved. However, perhaps research should be directed towards a 'panel' or 'multiplex' approach of techniques deployed simultaneously or sequentially. Such an approach, especially one using methods that detect different markers, would have the benefit of improving accuracy, as techniques which are sensitive to short term changes could be combined with those more sensitive to long term changes, providing a level of in-built cross-checking.

Some of the techniques discussed by the authors show very broad standard deviations which in some circumstances would result in a method lacking discrimination and ultimately being uninformative, depending on the time since deposition under consideration.

As the authors have highlighted, any proposed technique should ideally be portable enough for use at the crime scene (where it might be operated by non-scientists), as well as suitable for use in the forensic laboratory when examining blood stained exhibits. It may be of course that a two-tier approach may be developed, with a more 'presumptive' portable screening method for the crime scene, and a more accurate sophisticated method for the laboratory environment.

Regardless, it is important that the methodology can dovetail with the other tests that may be required, such as DNA analysis or (more rarely) blood species testing. The authors have usefully approached the topic in terms of how invasive the methods are, and whether the sample is consumed by the test. Frequently blood stains in casework are so small in size that not all the desired tests can be carried out, so any method that fully consumes the stain material (therefore preventing further, potentially more probative, testing) is undesirable.

As the authors correctly identify, a future blood ageing technique should also ideally utilise equipment that is already in forensic use, or would be inexpensive to purchase—a forensic organisation would be unlikely to invest in an expensive piece of equipment if its only application is ageing blood stains. Similarly, such an analytical technique would be unlikely to be introduced into a high-throughput workflow where case turn-round times are of the essence unless the technique is quick, and easily incorporated into the analytical process.

In conclusion, from a practitioner perspective, one of the most critical attributes of a prospective blood ageing method is perhaps compatibility of the technique

with further tests downstream, particularly DNA analysis. To that end, two fields of research discussed by the authors offer perhaps the most exciting opportunities.

Firstly, those blood ageing methods that utilise mRNA and miRNA degradation have an attraction in that there may be scope for co-extraction with the sample for DNA analysis, therefore limiting sample consumption, as well as potentially being compatible with existing analytical equipment at many forensic laboratories. Further work is undoubtedly needed though to identify additional RNA markers more suitable for identifying short term changes, and not just longer term degradation. Some of the metabolomics methods discussed by the authors, for example circadian hormone markers amalgamated with a suite of RNA markers, offer the intriguing prospect of identifying when during the 24 h day/night cycle the stain was deposited.

Secondly, reflectance spectroscopy and hyperspectral imaging, identified by the authors as being the most technologically evolved methodologies, have the advantages of being non-invasive, and requiring no sample preparation, whilst additionally they utilise portable equipment. As the authors point out, reflectance spectroscopy continues to have difficulties with blood stains on dark backgrounds and coloured substrates, all of which are commonly encountered at crime scenes and on items at the laboratory. Hyperspectral imaging may overcome this to some extent, particularly in the infra-red region, however the next challenge is developing the technology to handheld, user-friendly devices that can accommodate the difficulties of analysing blood stains at a complex crime scene where assorted upholstery, floor and wall coverings and textile substrates may be encountered, and validating the method with a wider range of adulterated blood stains.

Finally, it is worth adding that establishing time since stain deposition is not unique to bloodstains—it is a question similarly faced by the forensic practitioner when finding other body fluid stains at the crime scene or on items examined in the laboratory. For example, in a sexual assault where the victim might have had previous consensual activity with the assailant, showing that a semen stain on bedding was deposited around the time of the alleged offence and not in the days before could be critical. Consequently, studies of markers that occur in both blood *and* other body fluids, for example protein and enzymes that degrade as the stain ages, might therefore offer a wider forensic application than markers found in blood alone.

Chapter 9
Recent Technological Developments in MALDI-MSI Based Hair Analysis

Bryn Flinders, Tom Bassindale and Ron M. A. Heeren

Abstract Hair is a common piece of trace evidence found at a crime scene, however, often it is not possible to obtain DNA (due to the lack of a follicular root). These hair samples could potentially provide other intelligence, based on the molecular history of an individual that it contains. Currently, this type of analysis is performed using traditional hyphenated techniques gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). However, these techniques require a large amount of hair, not a few single strands such as those typically found at a crime scene and also involve extensive sample preparation. Recently new technologies such as matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) have been used to monitor the distribution of drugs of abuse in single hair strands. Using this technology it is possible to reveal the distribution of compounds in the hair more accurately and in single strands as opposed to milligram quantities required by traditional hyphenated methods. The use of MALDI-MSI could provide law enforcement agencies with lifestyle information on an individual and help to narrow down the pool of suspects.

Keywords Hair · MALDI-MSI · Drugs of abuse · Medication

9.1 Incorporation of Compounds into Hair

Hair is a fibre that covers a large portion of the body in mammals and originates from the follicle, which is located 3–4 mm below the dermis. The follicles are surrounded by a network of capillaries that supply blood to the growing hair. Hair grows in

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three phases; firstly it is in the anagen phase (active growing), then it moves into the catagen phase (transition) and finally the telogen (resting) phase. During the anagen phase hair grows from the bulb and this phase can last several years. The hair stops growing during the catagen phase and a keratinized club is formed in the follicle. This is then ready to fall out in the telogen phase and the process may then start again [1, 2]. During the growth of the hair, compounds are incorporated into the hair shaft. There are several ways in which this can occur, the most common route is via the bloodstream during the anagen phase of hair growth. Other methods include incorporation via sweat/sebum after hair formation and from environmental exposure (e.g. via contact with contaminated hands or smoking). It is also possible that the compounds can be incorporated into the hair from the surrounding tissue [3, 4]. These processes are illustrated in Fig. 9.1.

The hair shaft consists of three parts: (i) the cuticle, which makes up the exterior of the hair and is made up of overlapping scale-like cells, (ii) the cortex, consisting

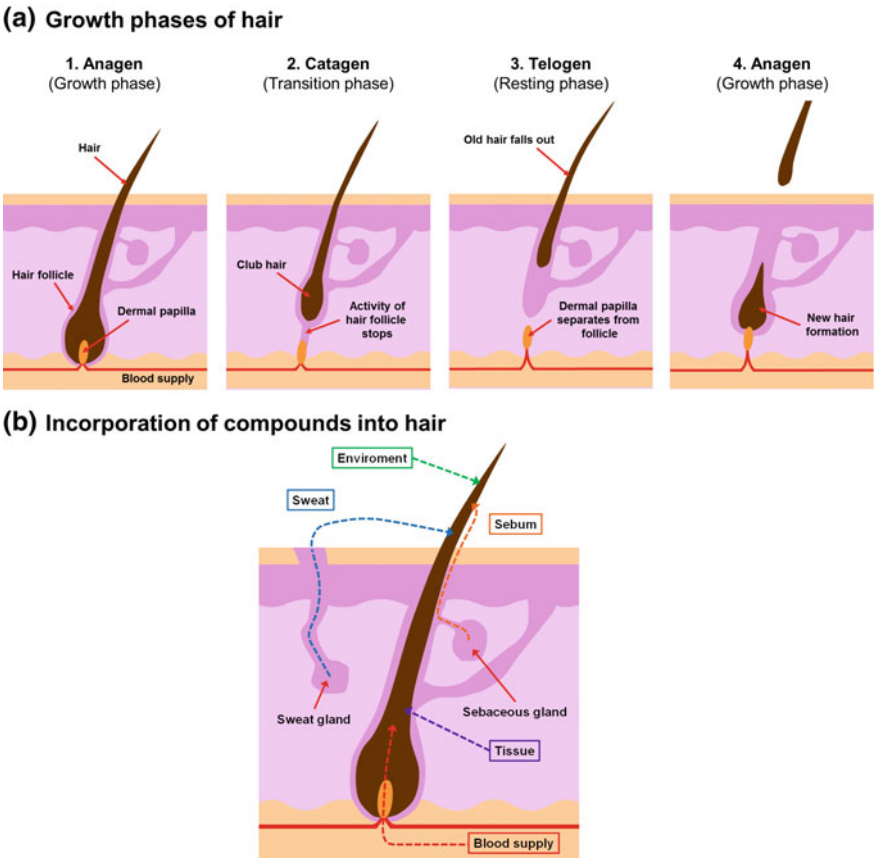

Fig. 9.1 Diagrams showing **a** the growth phases of hair and **b** incorporation routes of compounds into the hair

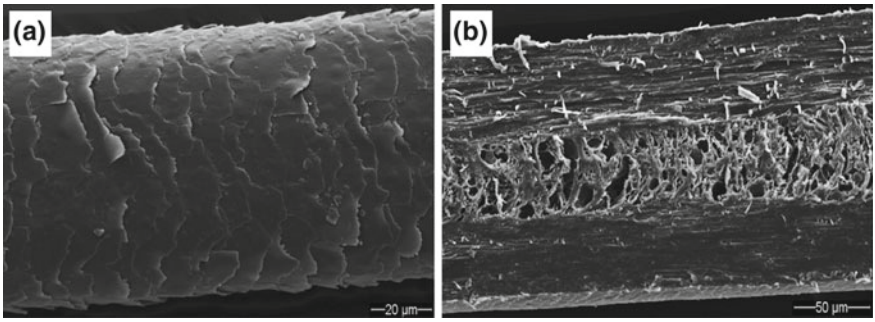

Fig. 9.2 Scanning electron microscope (SEM) images of hair samples. **a** Intact hair sample showing the exterior cuticle and **b** longitudinally sectioned hair sample showing the medulla surrounded by the cortex

of a fibrous structure and the location where the drug is bound and (iii) the medulla, at the centre of the hair consisting of a hollow honeycomb like structure [4]. Detailed images showing the different regions of hair are shown in Fig. 9.2.

Hair is common trace evidence found at a crime scene but usually in single strand quantities. In forensic science, nuclear DNA profiling of the follicular root of hair is often the best way to identify a suspect, however not all hairs found at a crime scene contain the root (falling out during the telogen phase). Mitochondrial DNA of the hair shaft is possible but cannot be used to definitively identify a suspect, unlike nuclear DNA and thus is of lower evidentiary value. Many forensic laboratories lack the facilities to perform this type of genetic test.

Microscopic comparative hair analysis is also a possibility, which involves comparing characteristics such as pigment distribution and scale patterns from hair of a suspect to those found at a crime scene. However this technique has come under a lot of scrutiny recently, which resulted in several convictions being overturned [5].

Hair testing for the detection of drugs of abuse is a powerful tool routinely used in toxicology and forensic applications as it offers several advantages of other biological samples such as prolonged detection window, which has clearly been demonstrated by the analysis of compounds such as cocaine in the hair of ancient mummies from civilizations dating back a millennia [6]. In forensic toxicology this gives us the opportunity to detect drugs in hair samples well after they have left the body, comparatively urine and blood only offer a short term exposure window (hours to days generally). In addition, hair sample collection is non-invasive and requires no special storage conditions like other biological matrices.

In this chapter, the feasibility of using MALDI-MSI in forensic analysis of hair is explored. The current applications, as well as the current limitations of the technique and its future in forensic hair testing are discussed.

9.2 Traditional/Current Methods of Analysis

Hair analysis is usually preceded by lengthy sample preparation procedures. Firstly, the hair sample may be segmented into the time period of interest; an estimation of 1 cm per month is used. Samples are often segmented into 1 or 3 month periods, but with the quantity required being 20–100 mg. Shorter hair samples require a thicker clump of hair consisting of hundreds of individual hairs. The general sample work up process involves: decontamination of the hair to remove external contamination, extraction of the drug from the hair to remove the drugs from the keratin matrix, sample clean up to preferentially extract the drugs of interest over matrix components (including steps such as derivatization) and finally analysis, which may involve reconstitution of the sample in the desired solvent. Vogliardi et al., conducted a comprehensive review of sample preparation methods for analysing drugs from hair samples [7]. To quantify the drugs present, a calibration curve is built by spiking known quantities of the drugs onto hair from non-drug users, to be extracted concurrently to the suspect hair samples. The use of internal standards is widespread, usually with deuterated analogues of the drugs of interest. Most drugs are present within the samples at levels of pg/mg to high ng/mg of hair, depending on the drug and its uptake into hair and the amount of drug used. The Society of Hair Testing has proposed some cut off values for commonly encountered drugs of abuse as well as some outline of procedures that should be followed in casework [8]. Hair analysis is used in a variety of applications amongst which: in child drug exposure cases, exploration of drug use trends, family court cases, pre-employment screening, following a driving ban and drug-facilitated offences [9–11].

Some of the initial analyses of hair used immunoassays to detect drugs [12–14]. Immunoassays are still in use but mainly found in high throughput laboratories where they are employed as a screening tool for classes of compounds such as opiates or benzodiazepines, prior to confirmatory analysis using a hyphenated technique.

Gas chromatography-mass spectrometry (GC-MS) has been used extensively to detect drugs from hair samples, usually employing an electron ionization source coupled to a single or triple quadrupole mass spectrometer. Due to the varied sample preparation methods required for different types of drugs, methods are often developed for drugs, such as cocaine [15, 16] amphetamines [17], opiates [18] or cannabinoids [19]. There have also been many methods developed for a more comprehensive screening using GC-MS [20–22]. The volatility of some compounds is too low to be analyzed via GC so sample derivatization is often needed, thus adding an additional sample preparation step; cannabinoids are an example of drugs requiring such treatment. Derivatization for GC-MS may also be required for other objectives such as to decrease fragmentation or to improve isomer separation (both examples pertinent to analysis of amphetamines). The use of hair samples to detect markers of alcohol consumption and abuse has also been proposed, with methods for ethyl glucuronide [23] and fatty acid ethyl esters [24] being available for GC-MS. Again, the Society of Hair Testing has proposed some guidelines for the interpretation of these results which is not without difficulty or debate [25].

The use of liquid chromatography-mass spectrometry has developed more recently with improvements over GC-MS. The absence of a derivatization step reduces sample work up and enables the analysis of many compounds in a single run. The development of multi-analyte screens, with many more metabolites incorporated into the method or indeed several drug classes being detected in a single run, has made hair analysis more appealing and versatile [9, 26, 27]. A much larger number of drugs can be detected from a single screen (>100 compounds) with high resolution mass spectrometry [28–30]. Further extensive reviews of the current analytical methods for hair analysis are available in the literature [31].

9.3 MALDI-MSI and Current Applications in Hair Analysis

Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) is a label free technique that can simultaneously monitor the distribution of drugs and metabolites. The technique also maintains the spatial localization of the compounds within a sample and can generate high spatial resolution images (<10 μm lateral resolution). The readers are directed to a series of review papers for a more detailed description of the technique and its various applications [32, 33]. A typical MALDI-MS imaging experiment of hair samples is illustrated in Fig. 9.3.

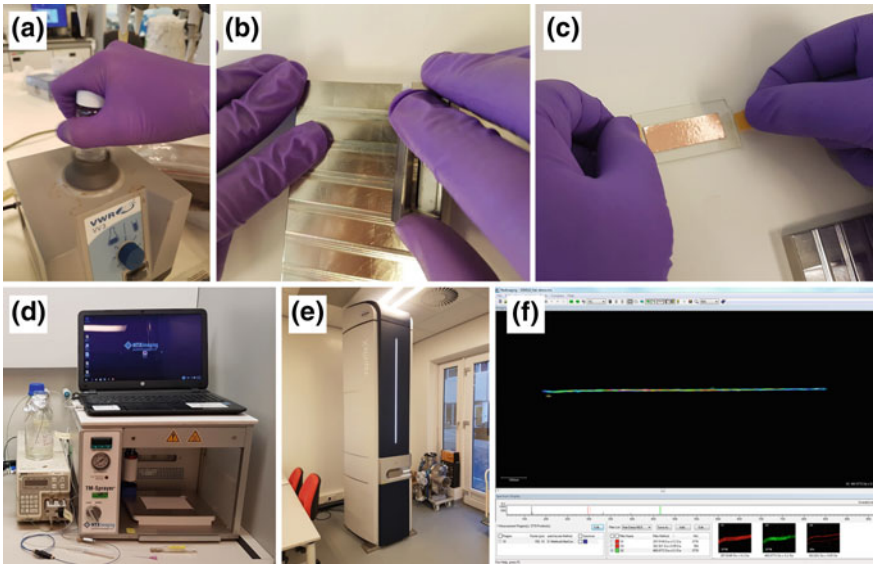

Fig. 9.3 Workflow for MALDI-MS imaging of hair samples. **a** Decontamination of hair samples, **b** longitudinal sectioning of hair samples, **c** mounting of hair samples, **d** matrix application, **e** MALDI-MS imaging and **f** data analysis and timeline visualization

As the workflow in Fig. 9.3 shows, the hair samples are first washed with one or a series of solvents (e.g. water, methanol or dichloromethane) to remove hair products, sweat and sebum. The washing step also removes other external contaminants such as any drug related compounds that could have been deposited from the environment. The hair samples are then longitudinally sectioned using a bespoke cutting device to expose the inside of the hair [34]. The hair samples are subsequently transferred and mounted onto a glass slide using double-sided conductive tape, after which the hair samples are coated with the MALDI matrix using an automated matrix sprayer (e.g. TM-sprayer, HTX technologies). Analysis is then performed using a MALDI-MS instrument (e.g. RapifleX, Bruker Daltonics) and images are constructed using vendor specific software (e.g. FlexImaging, Bruker Daltonics).

An advantage of MALDI-MS imaging of hair is the simpler and faster sample preparation, which can be accomplished in around 1 h. Traditional sample preparation time can run into days with the requirement to extract the drug from the hair and often perform lengthy clean up protocols. Another advantage is the improved time frame for compound detection that can achieved; the spatial resolution along the length of the hair determines the time frame. For example, a pixel size of 150 μm is equivalent to 11 h of growth, which is far more accurate than the current technique that provides this information per month. It is also of note that for MALDI-MSI the instrumental spatial resolution and sensitivity are continuously improving. The smallest pixel size that can be obtained with current state-of-the-art MALDI-MSI instrumentation (e.g. RapifleX, Bruker Daltonics) is ~5 μm [35], which corresponds to a growth time of approximately 0.36 h (22 min).

9.3.1 Drugs of Abuse

Over the last decade MALDI-MSI is increasingly being used for the analysis of hair and by far, the most common application is monitoring the distribution of exogenous compounds such as drugs of abuse in hair samples.

Vogliardi et al., initially explored the feasibility of using MALDI-MS for the analysis of drug of abuse in hair, in order to detect cocaine and its metabolites in authentic user hair samples. The authors used a sample preparation similar to that employed by the established analytical techniques, e.g. pulverization and extraction [36, 37]. The extract was then spotted onto a target plate treated with graphene and coated with the matrix α-cyano-4-hydroxycinnamic acid (CHCA) using an electrospray deposition system. Analysis showed the presence of cocaine and its metabolites benzoylecgonine and cocaethylene. Using this method a limit of detection of 0.1 ng/mg could be achieved for cocaine in the hair matrix. Whilst no imaging experiments were performed, and hence only limited temporal information was obtained, this work demonstrated the feasibility of detecting drugs of abuse from hair samples by MALDI-MS and laid the foundation for future imaging experiments.

Following this, Miki et al., monitored the distribution of methamphetamine in longitudinal hair sections obtained from a chronic user [38, 39]. Manual longitudinal

sections of hair samples were prepared to gain access to the inside of the hair, where the drug is bound. The authors also attempted to cut hair samples with the use of a laser microdissection system, however this resulted in poor mass spectral quality possibly due to thermal degradation. MALDI-time-of-flight (TOF)-MS imaging was performed on the hair samples. The resulting image showed, as the authors described it a "barcode-like" pattern, which arises from repeated drug administration. MALDI-Fourier transform ion cyclotron resonance (FTICR)-MS imaging was performed to provide further confirmation on the identity of methamphetamine. The images showed the same pattern as previously seen in the MALDI-TOF-MS images and the exact mass and tandem mass spectrometry (MS/MS) confirmed the identity.

Porta et al., monitored the distribution of cocaine and its metabolites benzoylecgonine, ethylcocaine and norcocaine in single intact hair samples from chronic users [40]. The hair samples were washed and mounted onto a MALDI target plate, then manually coated with CHCA using a TLC sprayer. A dilution series of cocaine and its metabolites was prepared and spotted next to the hair samples in order to provide quantitative information. Deuterated versions of these compounds were added to matrix solution to act as an internal standard. MALDI-MS/MS imaging was performed using a MALDI-triple quadrupole linear ion trap equipped with a high repetition rate laser.

Shen et al., monitored the distribution of ketamine in single hair samples obtained from a chronic user [41]. The surface of the hair samples was gently scraped off using a scalpel to expose the inside for analysis. The matrix was applied manually to the hair before analysis by MALDI-FTICR-MS. The images obtained from four hair samples taken from the same donor, showed the distribution of ketamine throughout three of the four hairs analyzed, however only trace amounts were found in the second hair sample.

Kamata et al., investigated the incorporation rates of methoxyphenamine (an analogue of methamphetamine) into hair using MALDI-MS/MS imaging [42]. The analysis was performed at various time points on single longitudinal sectioned hairs from volunteers after a single oral administration of the medication. The images showed the drug was localized in the root region below the scalp (4 mm) after 24 h; the images of the hair samples 3–7 days post administration showed that the drug then began to migrate from the root region to the scalp surface region of the hair. After 2 weeks the drug was localized in the hair above the scalp surface and absent from the root region.

Beasley et al., monitored the distribution of Δ^9-tetrahydrocannabinol (THC) in authentic user hair samples by MALDI-MS/MS imaging following derivatization [43]. The authors utilized 2-fluoro-1-methylpyridinium toluenesulfonate (FMPTS), a pre-charged reagent, to improve the sensitivity and detection of a range of cannabinoids and their metabolites. Recently Kernalléguen et al., monitored the distribution of synthetic cannabinoids in hair samples from self-reported users [44]. Hair samples were longitudinally sectioned and analyzed using MALDI-MS/MS imaging without any derivatization.

9.3.2 Medication

Within the management of chronic conditions, compliance to medications is crucial for the maintenance of the patient's health. The ability to monitor a patient's compliance to medication over a long period would be a great benefit to health care providers. This was demonstrated by Rosen et al., who monitored the distribution of the antiretroviral efavirenz, which is used in the treatment of HIV. The distribution of the drug was monitored in hair samples from three HIV infected patients using infrared-matrix-assisted laser desorption electrospray ionization-mass spectrometry imaging (IR-MALDESI-MSI). Normalization of the data was accomplished using pyrrole-2,3,5-tricarboxylic acid (PTCA), an oxidation product of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which is a eumelanin biomarker to account for hair colour [45]. The advantages of this technique over conventional MALDI-MSI is that it does not require a UV MALDI matrix, which may interfere with analysis of low molecular weight compounds (<500 Da). A detailed description of the IR-MALDESI technique is reported elsewhere [46].

Poetzsch et al., monitored the distribution of tilidine (a synthetic opioid painkiller) in hair samples by MALDI-MS/MS imaging with complementary LC-MS/MS analysis [47]. Hair samples from children obtained from a forensic case involving suspected tilidine intoxication, as well hair from chronic users were analyzed. The images showed the compound was distributed throughout the hair samples even after a single dose, which led the authors to conclude this was most likely due to sweat contamination and incorporation into the hair matrix. Later the authors monitored the distribution of zolpidem in the hair of volunteers after a single dose using MALDI-MS imaging [47]. The images showed a band approximately 0.8 cm in length (~3.5 weeks of growth) a month after administration. Similarly Shima et al., monitored the distribution of zolpidem in moustache and head hair of volunteers after a single administration using MALDI-FTICR-MS imaging [48, 49]. The images showed the drug resided in the root area of the hair 24 h after zolpidem administration, after 35 days zolpidem was present further along the hair in a small band. Recently Wang et al., monitored the distribution of olanzapine in the hair of volunteers that had been taking the drug for 4 weeks [50]. The hair samples were collected and prepared for imaging by manually scraping the surface of the hair away. The authors used a novel matrix, esculetin, for analysis of the samples using MALDI-FTICR-MS imaging. A similar matrix was also used by the authors to monitor the distribution of methamphetamine [51].

An example illustrating the application of MALDI-MS imaging to detect medication in hair is shown in Fig. 9.4. Here, MALDI-FTICR-MS imaging was used to analyze hair samples obtained from an epilepsy patient, taking the anti-epilepsy medication carbamazepine (prescribed a daily dose of 800 mg). The image shows that the distribution of carbamazepine is consistent through the five hairs analyzed. This indicates that this patient is complying with their medication as prescribed. The image also shows the drug is absent from the control hair sample, which was obtained from a donor not using the medication. The slight variations in distribution

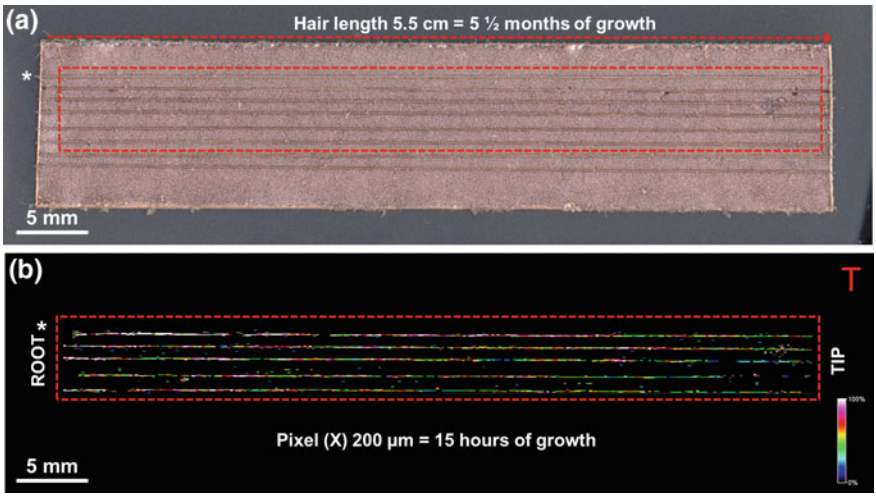

Fig. 9.4 MALDI-FTICR-MS imaging of carbamazepine user hair samples. **a** Optical image of longitudinally sectioned control and epilepsy patient hair samples following matrix application. **b** MALDI-FTICR-MS image showing the distribution of carbamazepine at *m/z* 237.1022. Analysis performed on the Bruker SolariX in continuous accumulation of selected ions (CASI) mode, spatial resolution of 200 × 20 µm and normalized with TIC (*control/drug free hair sample)

of the drug could be due to differences in growth phases or the preparation of the hair samples. Due to the use of a high mass resolving power instrument, the identity of the drug could also be confirmed.

9.3.3 Lifestyle Markers

Hair can also give an indication of personal lifestyle such as cigarette smoking. Nakanishi et al., demonstrated this opportunity by quantitatively monitoring the distribution of nicotine in single longitudinally sectioned hair from several heavy smokers [52]. Their work also shows that MALDI-MSI could be useful for confirming the cessation of smoking for health care practices.

9.3.4 Investigation into Forensic Procedures

MALDI-MS imaging has also been used to give insights into the effects of the decontamination procedure on the analysis of hair samples, as well as differentiate between external contamination and actual abuse. The consequences of different washing procedures on the distribution of cocaine in hair were investigated using MALDI-MS/MS and metal-assisted secondary ion mass spectrometry (MetA-SIMS)

imaging [53]. Intact hair samples that were externally contaminated with cocaine were washed with different solvents before analysis. The results showed that the most commonly used washing solvents, water and methanol, appeared to remove the most surface contamination. However, high spatial resolution imaging of longitudinal and cross-sections of contaminated hair samples showed the cocaine migrated into the hair giving rise to a false positive. If these samples had been analyzed by traditional methods they would have been called "a positive". This differentiation between external contamination and actual incorporation is only possible through the high spatial resolution analysis afforded by more advanced methods.

9.4 Current Challenges/Pitfalls of Hair Testing with MALDI-MSI

9.4.1 Growth Phases and Type of Hair

Whilst it has been demonstrated that the analysis of single hair samples is possible, one of the main challenges is the impact of the different growth phases on the interpretation of the results. As previously stated, hair undergoes cycles of growth and dormancy in three stages. The first is the anagen or growing phase which constitutes 85–90% of hairs, the catagen or transitional phase which is rare comprising only 1% of hairs and finally the telogen or resting phase consisting of 10–15% of hairs. The process then repeats and new hair begins to grow [4]. Variation in the distribution of a compound between hairs is often attributed to this but needs to be investigated more in depth. Another area that needs further investigation is the effect of the hair type on uptake and the distribution of a compound (e.g. arm, chest, leg or public hair), each having a different growth phase/rate. This is important as hair found at a crime scene may not always be scalp hair; for example in rape cases the most common hair type recovered is pubic hair. As such, the ability to interpret results from hairs other than head hair is very limited.

9.4.2 Sensitivity

There are several issues that can influence the sensitivity of detection of compounds in hair by MALDI-MSI. Unlike the established chromatography methodology, MALDI-MSI has no clean-up steps or separation prior to analysis. The latter can be compensated using ion mobility separation (IMS), which is accomplished immediately following ionization within the mass spectrometer. Several commercial instruments now have this analytical separation capability built into the instrument. The sensitivity is also associated with the efficiency of the extraction of the compounds from the hair. The sample preparation of currently established techniques

involves pulverization of the hair samples and the powder is then solvent-extracted with a procedure lasting from a few minutes to many hours. In contrast, MALDI-MS imaging of hair samples relies on local extraction of compounds directly from the hair, either through the cuticle of intact hairs or directly from the cortex of longitudinally sectioned hairs, which takes between 5 and 20 s and requires multiple passes or spray cycles using automated matrix sprayers prior to MALDI-MSI analysis. However, the preparation of the hair samples (e.g. intact vs. longitudinally sectioned hairs) for MALDI-MS imaging has an impact on the outcome. Longitudinal sectioning has been proposed to gain access to the centre of the hair, thus providing easier extraction of compounds [34, 38]. This is demonstrated in Fig. 9.5. As Fig. 9.5 shows, the exterior of the hair is coated with lauryl sulfate, an ingredient found in shampoos. The distribution of this species appears as two parallel lines in the longitudinally sectioned parts of the hair sample and clearly delineates the un-sectioned part of the hair. In contrast, the image of cholesterol sulfate (an endogenous metabolite) shows a higher intensity of the compound in the sectioned part, in comparison to the un-sectioned part. The overlay shows that these two species can discriminate between the interior and exterior of the hair sample. This example demonstrates the impact of the hair sample preparation on the detection of compounds and further illustrates the need for longitudinal sectioning of hair samples prior to the MALDI matrix application.

Some compounds have poor ionization efficiency, which limits the sensitivity of the method. In certain cases this can be overcome by derivatization prior to matrix

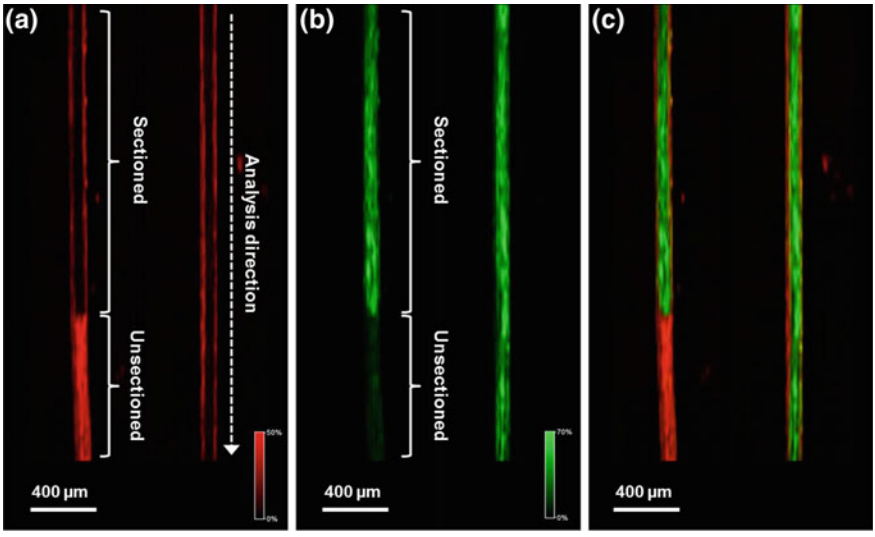

Fig. 9.5 MALDI-MS imaging of intact and longitudinally sectioned hair samples. MALDI-MS images showing the distribution of **a** lauryl sulfate at *m/z* 265.15, **b** cholesterol sulfate at *m/z* 465.30 and **c** overlay of selected species. Analysis performed on the Bruker RapifleX, spatial resolution of 100 × 10 µm and normalized with TIC

application. Beasley et al., recently demonstrated such a procedure with the analysis of cannabinoids in hair [43]. Interference from matrix peaks in the lower mass range (<500 Da) can also be an issue, however this can be overcome by targeted analysis such as tandem mass spectrometry (MS/MS), high mass resolving instruments (FTICR) or ion mobility separation.

9.4.3 Quantitation

Quantitation by MALDI-MSI is a topic of much debate in the field and as such, the methods used to provide the quantitative information vary across laboratories. All methods are typically complemented by established quantitation techniques. The most common quantitation method for MALDI-MSI involves spotting drug dilution series onto the glass slide next to the samples which are analyzed together [40]. However, this method does not accurately represent the response obtained from the sample, due to the difference in the matrix extraction efficiency of compound from the hair and the standards applied on the glass slide. Alternatively, spotting or spraying the dilution series onto control hair samples enables the calibration line to be matrix matched [54]. However, this method may not represent the matrix extraction efficiency. Ideally, the calibration curve should be matrix-matched and mimic the actual sample. Rosen et al., presented a method to prepare matrix matched standards, which involved incubating drug free hair samples in a dilution series of the drug [45]. The standards will also mimic the extraction of the drug from the actual hair samples with the MALDI matrix. However, the hair samples used to prepare the matrix matched standards need to be the same hair colour as the actual samples.

Hair can be considered a homogenous sample, the environment of which is not as complex as a tissue section. Poetzsch et al., demonstrated that the ionization of compounds is independent from the colour and the different structures of the hair. Therefore quantitation could be performed with or without an internal standard [55]. In contrast, Rosen et al., demonstrated that the hair colour does have an impact on the detection of compounds and developed a normalization method (using a melanin biomarker) to account for the variation [45]. This also correlates with the known phenomenon that darker hair bind compounds better than lighter coloured hair. However, the difference in findings is likely due to the different ionization methods employed in these studies. Regardless, if possible, the use of an internal standard is recommended to account for any signal variation and to normalize the images. Ideally, the internal standard should be an isotopically labelled version of the compound of interest or a structurally similar analogue. The internal standard can be incorporated into the MALDI matrix and homogenously applied onto the sample using automated matrix sprayers.

9.4.4 Limited Number of Compounds Detected

Despite the first demonstration of detecting compounds in hair being a decade ago and the increasing popularity of hair analysis with MALDI-MS imaging, only a selected number of compounds have been detected. In order to be implemented in forensic science, as well as other areas such as clinical screening to monitor patience compliance, the catalogue of compounds detected in hair needs to be expanded. A summary of the current compounds detected in hair is shown in Table 9.1.

As the table shows, the most commonly detected drug is cocaine, this is due to its high prevalence around the world, as well as its high ionization efficiency thus making it easy to detect. The table also shows that the most common MALDI matrix is CHCA, due to its versatility and sensitivity for the ionization of low to medium molecular weight compounds. Other MALDI matrices are being explored for hair analysis and as new matrices are being reported each year, the access to the detection new compounds is increasing. The table also shows that new compounds, such as synthetic cannabinoids, are now being detected in hair.

Table 9.1 List of compounds detected in hair by mass spectrometry imaging showing the matrix used and the limit of detection values as quoted by the authors (N/A indicates that no values were quoted in the referenced publication)

Compound	MALDI matrix	Instrument	Limit of detection	References
Cocaine and metabolites	CHCA	MALDI-MS/MS imaging	N/A	[54]
Cocaine and metabolites	CHCA	MALDI-MS/MS imaging	5 ng/mg	[40]
Cocaine	CHCA + graphite	MALDI-MS	0.1 ng/mg	[37]
Cocaine and metabolites	CHCA + graphite	MALDI-MS	N/A	[36]
Cocaine	CHCA	MALDI-MS/MS and MetA-SIMS imaging	N/A	[34]
Efavirenz	N/A	IR-MALDESI-MS imaging	1.6 ng/mg	[45]
Ketamine	CHCA	MALDI-FTICR-MS imaging	7.7 ng/mg	[41]
Methamphetamine	Umbelliferone	MALDI-FTICR-MSI	N/A	[51]

(continued)

Table 9.1 (continued)

Compound	MALDI matrix	Instrument	Limit of detection	References
Methamphetamine	CHCA	MALDI-MS and FTICR-MS imaging	N/A	[38, 39]
Methoxyphenamine	CHCA	MALDI-MS/MS imaging	100 fg/mm	[42]
Nicotine	CHCA	MALDI-MS/MS imaging	1.6 ng/mg	[52]
Olanzapine	Esculetin	MALDI-FTICR-MSI	N/A	[50]
Synthetic cannabinoids	CHCA	MALDI-MS/MS and MS^3 imaging	69 pg/mg	[44]
Tilidine	CHCA	MALDI-MSI	N/A	[47]
Zolpidem	CHCA	MALDI-FTICR-MSI	50 fg/mm	[48, 49]
Zolpidem	CHCA	MALDI-MS imaging	270 pg/mg	[55]
Δ^9-tetrahydrocannbinol	FMPTS + CHCA	MALDI-MS and MS/MS imaging	N/A	[43]

9.5 Conclusions and Future Prospective

The use of MALDI-MS imaging for hair analysis has been demonstrated with the currently reported work described in this chapter. MALDI-MS imaging has several advantages over the currently established techniques such as faster sample preparation, improved time frame of drug detection and visual representation of the data. The latter may become more and more important as it conveys a more accessible way for law enforcement agencies, court and juries to understand the data if presented as evidence. Even if MALDI-MS imaging may never replace the currently established techniques, it has been demonstrated that it can, at least, be a good complementary technique.

The application of MALDI-MSI to forensic hair analysis is now more feasible due to recent advances in matrix application devices that allow for fast and reproducible preparation of samples. Instrumental developments such as raster imaging [54] or new commercial instruments such as the RapifleX (Bruker Daltonics) [35] and more recently the uMALDI (Waters) source [56] now enable rapid analysis of samples. Whilst the cost of the instrumentation is an issue, this technology can be applied to other areas of forensic science, as shown in other chapters of this book. MALDI-MSI has already become routinely employed by other industries, such as the pharmaceutical industry for complementary analysis in drug discovery and development studies. Commercial screening of hair samples with MALDI-MS imaging is now available. This demonstrates a great interest in hair screening and a potential future market, which may branch out into forensics. Like all tools currently employed by forensic

scientists, MALDI-MSI will need to undergo further development and validation to mature into an invaluable and routinely used forensic tool.

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

Eva Cuypers

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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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.

Chapter 11
Emerging Approaches in the Analysis of Inks on Questioned Documents

Céline Weyermann and Korn-usa Techabowornkiat

Abstract Questioned document is one of the oldest fields of examination reported in forensic science. Documents are used as physical (nowadays sometimes virtual) traces of human transactions, thus questioning, falsification and counterfeiting certainly have existed since their invention and routine use. This is also the case for biblical texts and art pieces for which authenticity and authorship are often disputed. While mainly handwriting comparison was reported in early works, the composition and characteristics of inks on paper were often briefly discussed (see for example the early works of Demelle or Raveneau in the XVIIe). Since then, many technological developments have impacted questioned document examination, both with regard to the ink and paper production, as well as to the writing instruments or printing techniques. Nowadays, further progress have changed the world of (questioned) documents, through the introduction of virtual documents using electronic signatures and security documents such as passports using mixed physical and digital biometric data. Thus, the document examiner' expertise has to quickly evolve and adapt to such developments, sometimes necessitating the combination of skills from different disciplines not always co-existing in forensic laboratories (such as chemistry, physics, statistics, engineering, material science, computer science). After a brief overview of the historical development in both ink formulation and analysis, this chapter will investigate the relevance of rapidly evolving technologies for application to the examination of questioned documents in a forensic perspective.

11.1 Introduction

It is not possible to date the first human writings, but archaeological discoveries indicate that many different substances and instruments were widely used to write or carve

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inscriptions and drawings on different substrates such as stones walls. For example, in the Lascaux caves, metal oxides were used to produce the famous coloured paintings [1]. Later, writing inks used on papyrus and parchment were composed of carbonaceous compounds extracted from cephalopod (e.g. squids) or carbonized organic substances [2]. Since the early XII^{e} century, iron gall based inks have been widely used [3, 4]. At the time, the ink composition did not vary much (chemically and visually) and it was erasable only through scraping. Such inks were used well into the XX^{e} century [5]. Then, ink formulation evolved quickly to a variety of mixtures and properties, largely dependent on the development of different kind of writing and printing techniques. Thus, many ink compositions are available on the market and might differ significantly depending on the type of writing instrument (e.g. fountain, felt tip, roller ball or gel pens) or printer used (e.g. inkjet, toner or security printers). Inks are mainly composed of the following ingredients with some exceptions (e.g. toner printers do not require a solvent) [6, 7]:

- dyes and/or pigments (to colour the ink),
- vehicles (to solubilise the dye and apply it smoothly on the paper),
- resins and additives (to adjust the different ink properties).

The diversity of compounds used in each categories of ingredients, combined with production impurities, is particularly interesting in a forensic identification perspective [8]. In practice, ballpoint pen inks (developed in 1930s [2]) are still the most commonly encountered in caseworks, together with inkjet and toner printers.

Before discussing the emerging technologies for the analysis of ink on questioned documents, it is important to clarify the purposes of such analysis. The following issues can arise during forensic investigation and judiciary processes:

– ***Ink identification*** to determine the (type of) device at the source of a manuscript or printer text.
– ***Differentiation of ink entries*** to detect an added or modified text using a different writing instrument or printer.
– ***Ink traces enhancement*** to read erased, faded or latent ink lines on a document.
– ***Sequencing of ink line crossings*** to determine in which chronological order some texts were added to a document.
– ***Dating of ink entries*** to estimate the moment of apposition of a text on a document.

While identification of the source of an ink entry would be particularly useful in questioned documents, it is generally only possible to determine the type of ink and its main components depending on the selected examination techniques [8]. Thus, it is possible with a low magnification microscope to determine that a ballpoint pen was used to draw an ink line, while chromatographic methods will help determine that the dye basic violet and the solvent phenoxethanol were contained in the questioned ink. However, in practice, the brand, model and specific device remains generally unknown in the identification process [9]. Thus, it is rarely possible to determine from the examination of an ink entry that a *bic cristal* pen belonging to *Mrs X* was used to draw the questioned ink entry. This can be explained by the fact that inks are widely spread manufactured products evolving over time and across countries.

In fact, ink manufacturers might sell their products to several pen brands. Thus, many undistinguishable *bic cristal* pens were produced using the same ink bulk, which might have been used as well to produce other pen brands and models sold in different countries. In parallel, the *bic cristal* pen on the desk of *Mrs X* probably contains different colorants and solvents than the one she bought 10 years ago or than the one of her colleague bought at the same time in another country [10]. Such problems are typically forensic and cannot be entirely solved through technological developments.

Some criteria and general principles are also particularly important in forensic science to insure the quality of the applied and emerging methodologies [11, 12]:

- ***From general to particular***: observation to the naked eye will always precede further magnification using a microscope.
- ***From non-destructive to destructive***: it is important to minimise the alteration of the document as much as possible, so that the document can still be employed, remain readable, as well as to allow subsequent analyses (e.g. to increase knowledge or allow a counter-expertise). Thus, methods should be non-invasive (i.e. no alteration) or minimally invasive (i.e. only a minimal portion of the ink or paper is altered); this is why optical methods will generally precede chemical methods requiring an extraction.
- ***Reliability***: the precision and accuracy of the methods must be determined and minimised. It is particularly important to carry out replicate analyses to estimate the reproducibility of the technique (as well as the intra-variability of the examined ink specimens). Control samples, such as standards, must also regularly be analysed to calibrate the instruments.
- ***Selectivity***: the capacity of the method to discriminate between ink samples of different composition is particularly important to identify and compare ink entries. The discriminating power can be calculated using data acquired through the analysis of inks having the same source (i.e. intra-variability) in comparison to inks having different sources (i.e. inter-variability). Complementary methods, yielding a combined improved discriminating power, can be particularly useful.
- ***Sensitivity***: the limits of detection and quantification must be determined for qualitative and quantitative analysis, respectively. While a sensitive method might be considered an advantage to analyse small quantities of material, background will often increase proportionally with this criteria and reliability of the method might thus be a more important criteria.

Of course, the simplicity, rapidity, hazard, portability and cost of the methods are also important practical criteria, as well as the ethical appropriateness of its application and interpretation in a legal context. Taking into account all these criteria several methods can then be selected and applied in sequence to examine the ink traces with the objective to answer the questions asked in a specific casework. Thus, while optical methods will always be preferred in a first stage, emerging (potentially invasive) technologies might bring useful additional information in some particular cases.

11.2 Current Ink Analysis Methods

Together with the developments of ink composition and writing instruments, forensic methods have also evolved quickly during the past century. Of course, examination of documents was first carried out with the naked eye, and then using a magnifying glass which was certainly the first *technological* evolution in the examination of writings in questioned documents. The microscope is still a very important optical tool to observe and analyse all kinds of traces, including inks [5, 13]. While gallotannic inks remained difficult to differentiate based on optical observations, due to their very similar compositions, the question of artificial and natural aging was also discussed. Visible changes were reported such as the progressive darkening and cracking of the ink lines over time [3, 4]. Micro-chemical spot tests were also reported in the first questioned document manuals dealing with ink analysis [5, 13]. They were used to compare dissolution rates and colour changes by adding some solvents and reagents to the ink lines in a minimally invasive way using a microscope. Filtered light and spectroscopic examinations were also proposed by the same authors to compare inks entries in a standardised way. While optical and spectroscopic devices are still the main (and often, the only) instrumentation employed for questioned document examinations, micro-chemical methods are no longer used. In the 1950s, paper chromatography and electrophoresis were proposed to separate and identify dyes and pigments [14]. These have then been replaced by more advanced techniques such as thin layer chromatography (TLC), liquid chromatography (LC) and capillary electrophoresis (CE) during the second part of the XX^{e} century [15]. While many novel and highly technological techniques emerged these past years [16, 17], TLC and high performance (HP)TLC are still the most common (and probably the cheapest) chemical methods used in forensic laboratories around the world (Fig. 11.1). (HP)TLC results were implemented in two of the largest ink libraries in Germany and the USA [18–20].

Based on ink analysis methods reported in the literature [16, 17], a proposition of the most promising approaches for ink comparison is made here and discussed. Paper analysis was not considered in this chapter, neither were ink dating and ink line sequencing [17, 21, 22] as it is well known that the potential of optical methods remains very limited for the purpose of ink dating and sequencing. To date, the most promising technique relies on solvents analysis for ink dating purposes using gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) [23, 24]. For ink line sequencing, 3D laser profilometry might be an interesting complement to optical approaches. However, no methodologies was reported as validated yet for practical purposes [25].

While all kinds of potentially interesting technologies that can be useful for ink analysis and discrimination are reported in the literature, few studies actually reported large population studies. These would allow evaluating their actual capabilities and added value compared to traditional methods. A list of studies reporting the discrimination power of the techniques on various ink populations was compiled in Table 11.1. While most methods focused on the analysis of the visible coloured part

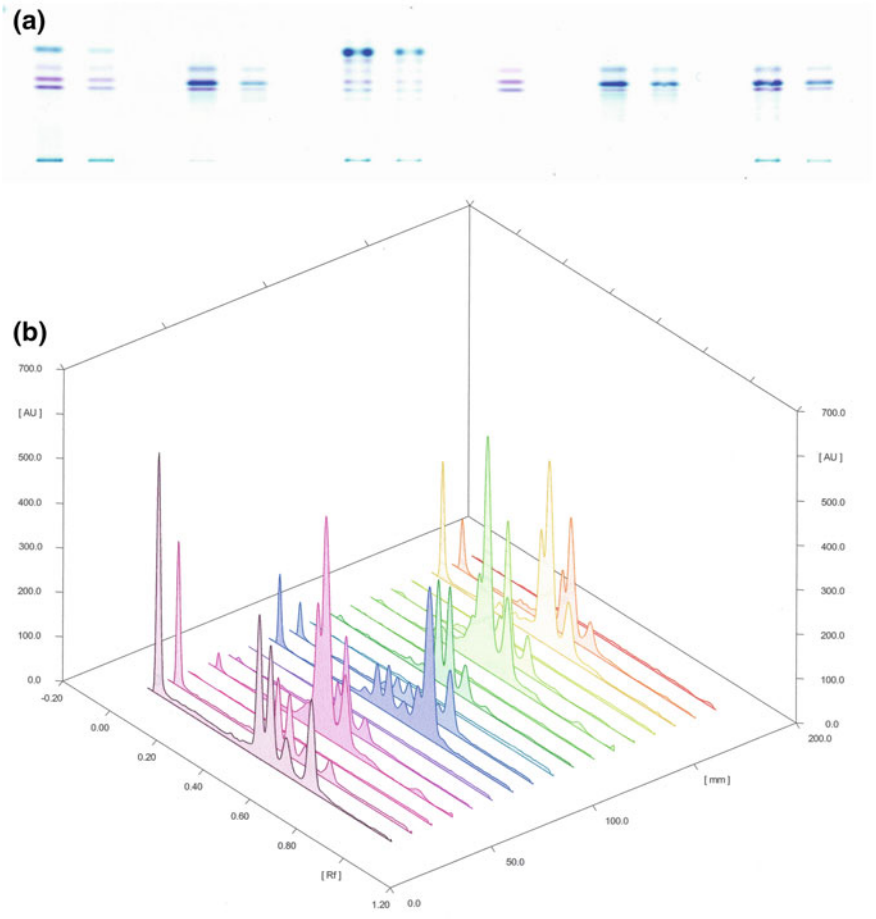

Fig. 11.1 HPTLC plate results for the analysis of 5 blue pen ink samples (each in duplicate) and one basic violet 4 reference using a CAMAG Linomat 4 TLC spot applicator (**a**) and a CAMAG TLC 3D scan to report the density as a function of the retention factor (Rf) for the 11 spots (**b**)

of the inks (dyes and/or pigments), others targeted resins, additives, solvents and/or elemental composition. As mentioned above, non-invasive or minimally invasive techniques will generally be preferred in practice. Thus, optical and spectroscopic techniques generally remain the most interesting alternatives in a forensic context.

After observation under different magnification, the first applied technique in questioned document examination is generally *filtered light examination* (*FLE*). It is generally available in all laboratories and video spectral comparators (VSC) were developed by several companies to facilitate and automate the use of this method. Results can be easily saved as images. This technique is often used for preliminary examination of questioned documents, and will allow direct comparison of the optical properties of ink entries through the combination of different illumination and

Table 11.1 Selected researches mentioning the discriminating power (DP) of the evaluated techniques. Costs was indicated in a comparative way including maintenance (depending on the instrument brand, model and options, the actual costs can significantly vary). Simplicity is rarely mentioned in the literature and was indicated based on the knowledge and experience of the authors (and may also vary depending on the instrument options and evolution). BP, GP, IP and FI means ballpoint pens, gel pens, inkjet printers and fluid inks, respectively. (*S*) indicates the use of statistics to calculated the indicated discriminating powers. As can be seen, statistical treatments were more frequently applied on data acquired by advanced technologies. References were found from several sources, such as Scopus, the university library and review articles.

Techniques	Type and # of inks	Discriminating power (%)	Invasive	Costs	Complexity	References
FLE	49 blue BP	83	No	Minimal	Minimal	[26]
	42 black BP	96				[26]
	30 black GP	49				[27]
	15 black GP	68				[28]
	50 blue BP	> 95 (S)				[29]
MSP	49 blue BP	83	No	+	+	[26]
	42 black BP	83				[26]
	30 black GP	74				[27]
	12 blue BP	79				[30]
	21 black BP	96				[30]
HSI	15 blue GP	90	No	+	++	[31]
	14 black GP	38				[31]
	45 black GP	60 (S)				[32]
(Micro) Raman	15 black GP	77	No	++	++	[28]
	36 blue GP	68				[33]
	17 blue GP	89				[34]
	14 blue BP	98 (S)				[35]
	23 coloured IP	94				[36]
	27 blue BP	92				[37]
	23 black BP	91				[37]
	26 blue BP	47				[38]

(continued)

Table 11.1 (continued)

Techniques	Type and # of inks	Discriminating power (%)	Invasive	Costs	Complexity	References
	26 black BP	58				[38]
	63 blue FI	47				[38]
	60 black FI	74				[38]
	16 blue BP	88				[39]
SERS	15 black GP	90	Yes	++	+++	[28]
	26 blue BP	80				[38]
	26 black BP	33				[38]
	63 blue FI	83				[38]
	60 black FI	92				[38]
	13 blue FI	75				[40]
(ATR) FTIR	27 blue BP	90	(Yes) no	++	++	[37]
	23 black BP	94				[37]
	8 blue BP	63 (S)				[41]
	54 toners	90				[42]
TLC	49 blue BP	98	Yes	Minimal	Minimal	[26]
	42 black BP	99				[26]
	16 blue BP	96				[39]
	7 blue BP	87				[28]
	10 blue BP	82				[43]
	9 black BP	81				[43]
	41 blue BP	83 (S)				[44]
HPTLC	31 blue BP	92	Yes	+	+	[45]
HPLC	8 blue BP	98 (S)	Yes	++	+++	[41]
LDI-MS	31 blue BP	99	Minimal	+++	++	[45]
	33 blue BP	99				[46]
	33 blue GP	92 (S)				[47]
	30 blue GP	85 (S)				[48]
	10 IJ	95 (S)				[49]
DART-MS	28 blue BP	100 (S)	Minimal	+++	++	[50]
	48 black BP	99 (S)				
	19 blue GP	97 (S)				

(continued)

Table 11.1 (continued)

Techniques	Type and # of inks	Discriminating power (%)	Invasive	Costs	Complexity	References
	21 black GP	93 (S)				
LIBS	34 blue inks	83	Minimal	++	++	[51]
	30 black inks	82				[51]
	11 black GP	96 (S)				[52]
	21 black IJ	94 (S)				[53]
	27 black toners	89 (S)				[53]
(LA) ICP-MS	21 blue BP	96 (S)	Yes	+++	+++	[54]
	22 black BP	99 (S)				[52]
	29 black GP	98 (S)				[52]
	21 black IJ	98 (S)				[53]
	27 black toners	100 (S)				[53]
ToF-SIMS	24 blue BP	91 (S)	Yes	+++	+++	[55]
GC-MS	32 blue BP	94	Yes	++	++	[56]
(LA) Py GC-MS	8 black toners	100 (S)	Yes	+++	+++	[57]

Costs was indicated in a comparative way including maintenance (depending on the instrument brand, model and options, the actual costs can significantly vary). Simplicity is rarely mentioned in the literature and was indicated based on the knowledge and experience of the authors (and may also vary depending on the instrument options and evolution). BP, GP, IP and FI means ballpoint pens, gel pens, inkjet printers and fluid inks, respectively. (S) indicates the use of statistics to calculated the indicated discriminating powers. As can be seen, statistical treatments were more frequently applied on data acquired by advanced technologies. References were found from several sources, such as Scopus, the university library and review articles

observation wavelengths (from ultraviolet to infrared light). Significant differences such as luminescence compared to absorption or transparency can be easily interpreted, while slight differences of the same optical characteristic (strong compared to medium luminescence) will be more challenging to evaluate as they may also be due to other influencing factors such as a slightly altered pressure while writing (Fig. 11.2).

The evaluation of the observed optical properties of the inks under different illumination and observation filters remains mainly visual and subjective. Therefore, only few quantitative results have been reported on the discriminating power of such approaches. Four studies reported discriminating power between 49 and 99% for

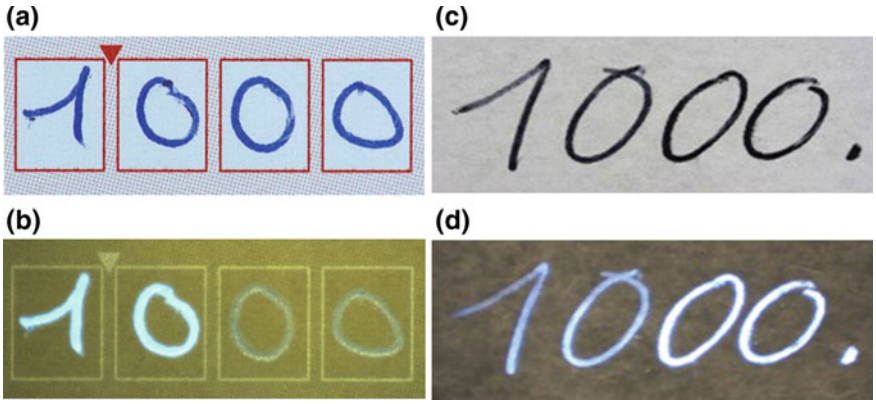

Fig. 11.2 Blue and black ballpoint pens were used to draw the numbers "1000" on paper and observed using a Docucenter Nirvis PIA7000 from Projectina (Switzerland): **a–c** images taken under white light, **b–d** images resulting from excitation at a wavelength range of 550–650 nm and emission at 735 nm. The difference of fluorescence observed in the blue pen image **b** clearly indicate that two pens were used, while the slight difference of fluorescence in the black pen image **d** might not suffice to indicate that two different pens were used as it might also come from a stronger pressure applied in the writing of the last two digits

ballpoint and gel pens [26–29]. While the discriminating power obtained for gel inks are lower than those obtained for ballpoint inks, the differences might also be due to different sample size and origin. A recent study proposed to evaluate the potential of FLE using chemometrics[1] to discriminate 50 blue ballpoint pen inks [29]. Different combinations of illumination and observation settings were selected statistically and yielded a discriminating power above 95%. Improving the image classification through automated systems, or using semi-quantitative spectroscopic approaches such as *microspectrophotometry* (*MSP*) or *hyperspectral imaging* (*HSI*) combined to statistical data treatment might represent the most efficient emerging technologies for ink analysis and comparison (Fig. 11.3). Such approaches are non-invasive, relatively simple and easy to automate. They also remain economic and selective as demonstrated by the reported discriminating powers in Table 11.1.

The creation of a shared database would be feasible using the same validated and calibrated method between the different laboratories feeding the system [58]. Reported discriminating power for MSP ranged from 74 to 96% applying visual comparison of the obtained UV-VIS [26–28]. Recent developments proposed to add a spatial component to spectroscopic data obtained, through HSI [59]. While the spatial dimension is not per se useful for ink analysis, it has the advantage to directly take into account the surface irregularities that can occur on the surface of an ink entry due to its deposition on paper (and thus characterise the intra-variability of the inks' spectroscopic response). One study proposes a statistical methodology to discriminate two inks on a document using HSI [58]. However, only 5 blue and 5

[1]Chemometrics is the use of statistical methods to extract useful information from chemical data.

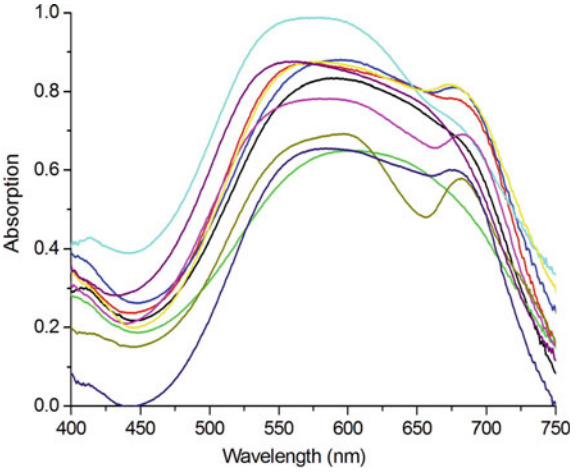

Fig. 11.3 Mean absorption spectra of 10 blue ballpoint pen entries acquired using a Zeiss Axioskop instrument equipped with an Epilon—Neofluar objective 20x/0.50 HD

black pens were evaluated (for a total of 10 possible combinations for each colour). While the proposed algorithmic approach might be interesting, it remains complex and still far from forensic implementation. More inks should be analysed to evaluate the real potential of HSI. Two studies did report a discriminating power from 40 to 90% for the analysis of gel pens inks using HSI [31, 32].

Other very interesting (to some extent non-invasive) technological approaches are offered by *Raman*[2] and *infrared* (*IR*) *spectroscopies* (Fig. 11.4). Gel pen inks and toners cannot be analysed using HPTLC as they generally do not contains dyes, but pigments (that are insoluble in the solvent systems used in HPTLC). Thus, Raman was proposed to analyse gel pen inks and inkjet printers [60–62], while Fourier Transform (FT)-IR was applied to discriminate toners [63, 64]. Surface Enhanced Raman spectroscopy (SERS)[3] was recommended to analyse dye-based inks in order to avoid fluorescence and enhance Raman signals [38]. Due to the small amount of reagent applied to the ink entry, the SERS technique can be considered as minimally invasive. Raman microspectroscopy and the use of multiple laser wavelengths were also suggested to increase performance and discrimination. Discriminating powers from 47 to 98% were reported in the literature [28, 33–38, 40]. Two studies concluded that the discriminating power was generally significantly higher using SERS [28, 38].

Again the highest discriminating power was obtained for data treatment using chemometrics. Only one study reported a discriminating power for toner analysis

[2] In brief, the Raman effect is a change in the wavelength of light occurring when a light beam is deflected by molecules, thus yielding information on the structure of the targeted molecules.

[3] SERS allows to increase the RAMAN signal of several orders of magnitude (and thus, the sensitivity if the technique) through a particular sample preparation that allow molecules to be adsorbed on metallic surfaces typically made of gold or silver.

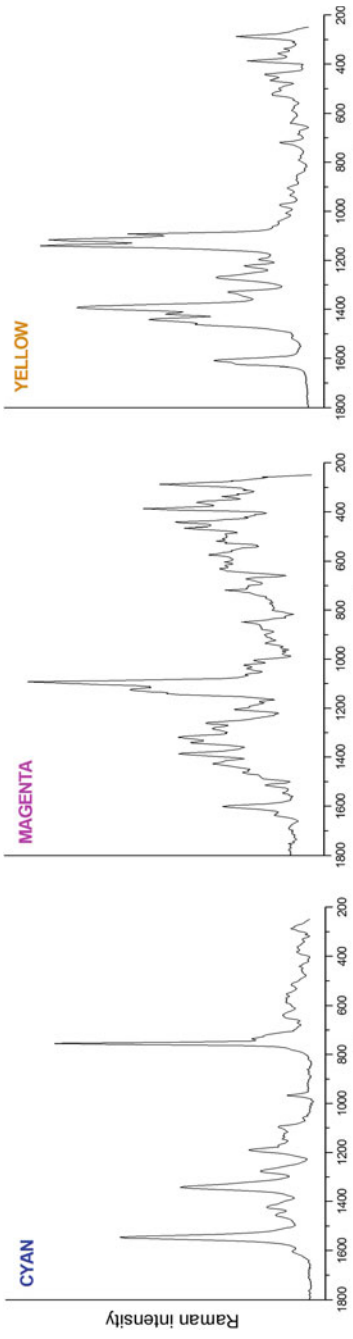

Fig. 11.4 Raman spectra of the cyan, magenta and yellow components of the ink from an inkjet printer. Spectra have been acquired using a Horiba Scientific instrument equipped with a diode-array near-infrared laser at 785 nm [62]

using micro Attenuated Total Reflectance (ATR)[4]-FTIR and statistical treatments reaching 90% for 54 analysed samples [42]. Two other studies focused on the analysis of ballpoint inks [37, 41]. Interestingly, the lowest discriminating power (63%) was obtained for 8 blue inks spectra compared through statistical treatments using replicate analyses from different pens of the same brand. This indicates that taking into account the statistical variability of a given ink (i.e. the intra-variability of the specimens) might reduce significantly the discriminating power.

Separation techniques are generally used only if optical and spectroscopic techniques did not allow ink discrimination. *Thin layer chromatography* (*TLC*) is generally available in all forensic laboratories. It is a very cost effective and simple method, showing a very good discriminating power from 81 to 99% [26, 28, 39, 43, 44]. HPTLC is a technological development that allows automatic sample deposition on the high performance plate. The chromatographic analysis is followed by an automatic scan yielding a densitometric analysis (Fig. 11.1) either in absorbance or luminescence mode [45]. This setup increases the cost and complexity of the analysis in parallel to the reliability of the results. One major drawback of HPTLC is that, as already mentioned, it does not work for pigmented inks and necessitates up to 1 cm ink to be extracted from the document. Thus, it is an invasive technique that requires authorization from the mandator. Finally, it was observed that the creation of a library has to be controlled using strict standardized conditions and references, as the elution distance can vary significantly between runs and was thus not always reliable [18].

Other more advanced techniques were proposed such as *high-performance liquid chromatography* (*HPLC*) and *capillary electrophoresis* (*CE*) using different detectors. Pigments and solvents can thus be analysed using the same technique. Even resins can be detected using advanced *ultra-performance LC coupled to a MS detector* (*UPLC-MS*). However, to date only one study reported a discriminating power of 98% using HPLC [41]. *Gas chromatography coupled to a mass spectrometer* (*GC-MS*) is used for the analysis of volatile compounds such as ink solvents. While solvents decrease rapidly in the ink entries, it can be used for ink differentiation as indicated by the discriminating power of 94% obtained from the qualitative data reported for the analysis of 32 ballpoint pen inks [56]. Adding quantitative determination of the solvent amount in the ink line might increase the potential of the method for ink discrimination. Thermal desorption (TD) GC/MS can furthermore detect resin compounds [65].

Several mass spectrometry (MS) techniques were also applied to ink analysis [17]. *Laser desorption ionization* (*LDI*) and *direct analysis in real time* (*DART*)-*MS* enable the mass spectral identification of key ink components (Fig. 11.5) and discriminating power up to 99% and minimal invasiveness were reported in the literature [45–50]. However, the use of chemometrics to treat the data taking into account the intra-variability (i.e. the variability among specimens coming from the same source ink)

[4] ATR is a sampling technique using a crystal to reflect the infrared beam of light. It allows measuring surface properties with no sample preparation and for absorbing material it additionally increases the IR signal.

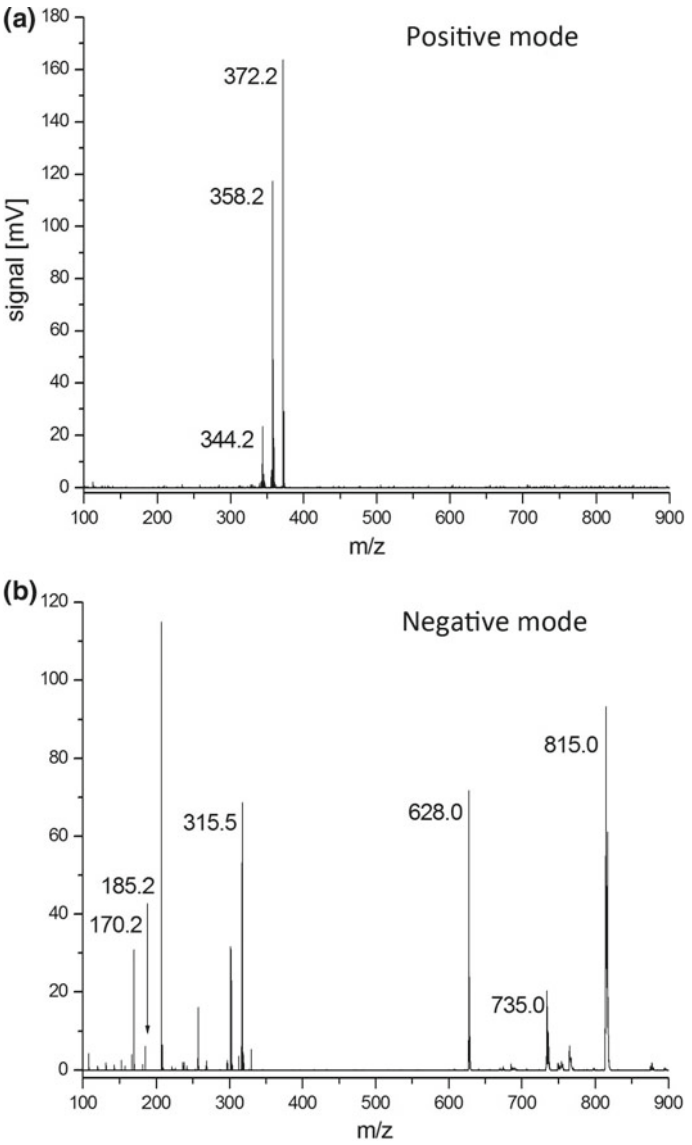

Fig. 11.5 LDI spectra of a blue ballpoint pen entry analysed directly on a piece of paper [46]. Basic violet 3 (*m/z* 344.2, 358.2 and 372.2) was identified in the positive mode (**a**), while acid blue 9 (*m/z* 170.2 and 185.2), acid blue 92 (*m/z* = 313.5 and 628.0) and solvent blue 38 and its mono-sulfonic acid derivative (*m/z* 735.0 and 815.0) were detected in the negative mode (**b**)

tend to show slightly lower (and probably more realistic) discriminating power values (Table 11.1).

Finally, elemental composition analysis was additionally evaluated for ink discrimination [17]. *X-ray fluorescence* (*XRF*) is generally more often available in forensic laboratories, but no studies calculated a discriminating power using this method. *Laser induced breakdown spectroscopy* (*LIBS*) yielded discriminating power from 82 to 96% [51–53]. More advanced methods were applied to ink differentiation such as *laser ablation inductively coupled* (*LA-ICP*)*-MS, time-of-flight secondary ion* (*ToF-SI*)*-MS, Laser ablation pyrolysis* (*LA-Py*) *GC-MS or Scanning Electron Microscopy-Energy Dispersive spectroscopy* (*SEM-EDS*).

While SEM-EDS yielded really poor discriminating powers, the other techniques allowed from 90 to 100% discrimination using chemometrics [53–55]. However, these methods are very complex, expensive and to some extent invasive. They will generally not be available in forensic laboratories.

An interesting emerging technology for ink analysis might reside in the coupling of *Raman* and *SEM-EDX*, combining to kind of information in one experimental design [66]. However, it was not tested yet for ink analysis purposes.

11.3 Emerging Techniques

While many advanced techniques have been listed and discussed above, two kind of emerging technologies can be highlighted as particularly promising for ink analysis in view of the evolution of ink formulation.

Optical and spectroscopic methods, such as ***HSI***, ***Raman*** *and* ***IR spectroscopy*** have the advantage of meeting most of the constraints within an operational forensic context: they are non-invasive, reliable, selective, sensitive, simple, rapid, economic and safe for the health of the examiners. Some instruments can even be transported on a crime scene and offer imaging capabilities [59, 67–72]. Spectra can easily be stored in a database and shared by forensic laboratories at the condition that the instrument measurements are calibrated using certified standards to insure comparability of the results [73]. While visual comparison of the spectra was generally used in routine work, the amount of data generated by imaging techniques will necessitate powerful data treatment software and chemometrics in order to visualise and compare results (Fig. 11.6). Raman and IR spectroscopy are often already available in forensic laboratories for other applications such as paint, drugs or fibre analysis, and thus can be easily transferred to ink analysis. HSI modalities are now directly integrated in the latest models of Video Spectral Comparator, an instrument that is routinely used by questioned document examiners around the world [71]. As shown in Table 11.1, all three techniques showed very promising discriminating power and are, to some extent, complementary in terms of targeted ink type. HSI might be less discriminating, but more versatile. Raman will generally be preferred to analyse pigment containing ink entries made for example from gel pens or inkjet printers [33, 62], and IR spectroscopy will be particularly adapted to inks containing resins such

as toners [74]. Portable instruments will also have the advantages of being easier to use and thus implementable in smaller forensic laboratories at reduced costs. The resulting discriminating power might be lower, but the portability developments are expected in the near future to make these instruments a viable alternative to present examination techniques.

If the discriminating power has to be increased, analytical chemistry *methods might be considered in a second analytical stage*. Amongst them, the least invasive would be mass spectrometry techniques using minimally invasive desorption. **LDI-MS** was first proposed for ink analysis [75] and showed promising discriminating power (Table 11.1 and Fig. 11.3). As vacuum is needed to ionize the samples, ink entries generally have to be cut from the paper and inserted in the ionization chamber, where solvents will be rapidly evaporated. Even so, LDI-MS can be considered as being minimally invasive, as the ink line can still be analysed to detect colorants and additives using LDI-MS or HPTLC. The use of a matrix (MALDI) might increase the selectivity and sensitivity of the method, but it would add to the invasiveness of the sample preparation [49]. The emergence of ambient ionization MS represents a very interesting development in terms of minimising invasiveness as the ink is sampled directly from a document substrate without the need to cut out the paper substrate and showing no or minimally visible alteration. **Direct electrospray ionization** (**DESI**), **Direct Sample Analysis** (**DSA**) and **Direct Analysis in Real Time** (**DART**) are different types of ambient ionisation options [50, 76–78]. DESI was first reported and involves a solvent being electrosprayed to generate charged droplets that are directed at the ink sample directly on paper [76]. While LDI and DESI allow imaging of the

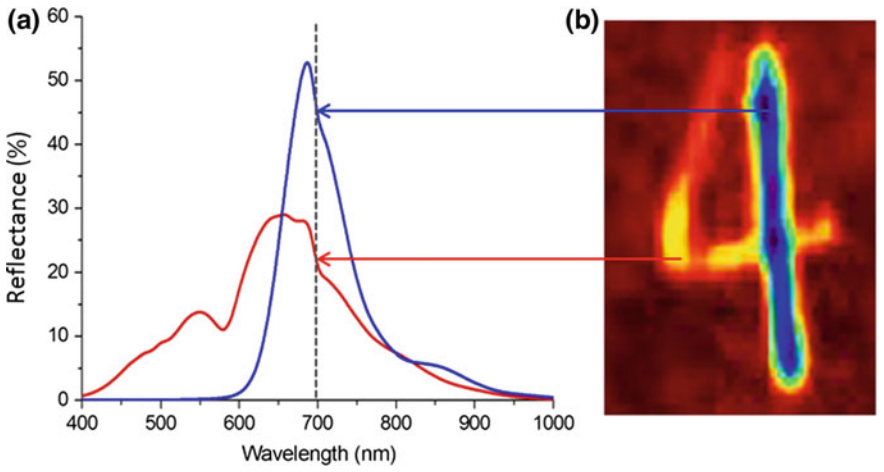

Fig. 11.6 The number four was drawn using two blue ballpoint pens: **a** Reflectance spectra acquired using a Docucenter Nirvis PIA7000 from Projectina. **b** Hyperspectral imaging at wavelength 700 nm acquired using a Spectral Camera sCMO from SPECIM

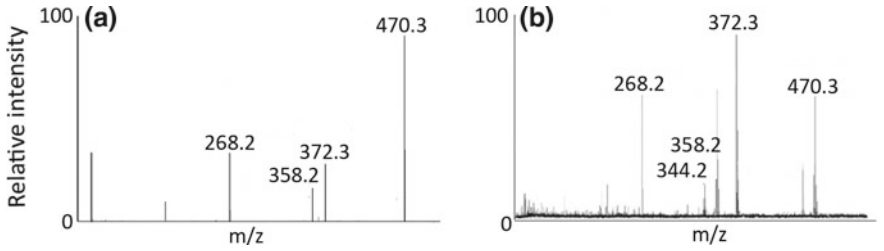

Fig. 11.7 DSA (**a**) and DART (**b**) mass spectra of 5 mm blue ballpoint pen ink entries on paper. DSA analysis was performed on an AxION DSA in conjunction with an AxION 2 time of flight mass spectrometer from Perkin Elmer [78]. DART analysis was acquired with a DART 100 ion source from IonSense coupled to an AccuTOF JMST100LC mass spectrometer from JEOL and showed more background noise [77]. Michler's ketone (m/z = 269.4), basic violet 3 (*m/z* 344.2, 358.2 and 372.2) and basic blue 7 (*m/z* 470.2) were identified by both techniques

samples, DESI was not further applied to the analysis of ink. This might be due to the fact that it is less user-friendly and sensitive than LDI-MS. Thus, the potential of other ambient MS technologies were tested and compared. DSA[5] and DART[6] actually produce similar ink spectra (Fig. 11.7) [77]. While DSA-MS generates less background signal, the open source configuration of DART-MS provides more flexibility for sample positioning and size, thus yielding a better sensitivity. Both techniques showed comparable repeatability. In comparison to LDI-MS, smaller molecules including more volatile compounds dominate the acquired mass spectra. This represents both an advantage and a disadvantage. Indeed, the techniques are less invasive and can detect ink solvents additionally to dyes. However, the ink composition profiles are modified over time as solvents evaporates [50]. As long as the ink samples are compared at the time of on the same document, this does not represent a major issue. On the contrary, age differences might even highlight an addition made with the same pen but at a different time (Fig. 11.2). However, this is also a hindrance to the implementation of a database as different mass spectra can be acquired over time for the same ink. Thus, it necessitates the identification of peaks in the spectra (i.e. ink components) showing stable relative areas, that will be extracted to feed into the database. This task can be automated using chemometric approaches [79]. Machine learning algorithms might thus represent a promising development for the implementation of such techniques in practice. The main drawbacks of these emerging technologies lie in the costs and size of such instruments. Indeed, while the initial costs might already be an issue, the annual maintenance and consumable costs will generally not justify the purchase of such equipment solely for questioned document examination.

While these emerging techniques represent very promising alternatives to presently used optical and chemical techniques, no reported study demonstrated that they actually yield better results in a forensic context yet [26]. This is due to

[5]DSA uses a ion gun that direct heated nitrogen gas molecules at the samples in a mesh holder.

[6]DART uses stream of excited helium or nitrogen to ionize samples directly on the paper substrate.

the fact that most studies calculate the discriminating power of a set of ink samples from different brands bought on the market. There is actually no indication that the bought inks are really different or represent inks that would be encountered in forensic practice. Thus, discriminating powers calculated in the literature are only indicative and cannot be compared to those obtained in other studies (except when the same ink populations were used). In fact, calculated discriminating power might be influenced by the size of the ink population (i.e. the more, the better), by the selected samples (i.e. different type of inks, colours, brands, models), by the different techniques and experimental settings and finally by the different ways to compare results (i.e. qualitative or statistical approaches).

The fact that specimen variation is rarely taken into account in the discriminating power calculation is also a major concern in forensic science. It is essential to insure that the intra-variability of the ink specimen (i.e. the variability among specimens originating from the same ink) is significantly lower than the inter-variability (i.e. the variability between specimens originating from different inks) [48, 80]. The discriminating power of studies taking into account the intra-variability often yields lower and more realistic discriminating power. Additionally, the development of objective comparison approaches (i.e. using transparent and easy-to-use statistical approaches) will also yield more reliable evidence for court purposes in comparison to subjective comparison of luminescence or TLC plate. The main remaining challenge will then be to present to a Court Raman or LDI results in a similar straightforward way than the luminescence images presented in Fig. 11.2 or the TLC plate presented in Fig. 11.1.

11.4 Conclusion

As it can be seen, despite the tremendous progress in technologies made in these last years and the manifold techniques proposed in the literature [16, 17, 81, 82], most forensic laboratories still use very simple and economic techniques. One can ask why most forensic laboratories have not implemented these developments in practice. Several reasons can be suggested. From the personal experience of the authors, working in two operational forensic laboratories in Switzerland and Thailand, casework necessitating ink analysis, comparison or dating represent only a small part of the questioned document examinations compared to handwriting comparison or identity documents inspection. Even the examination of documents to detect potential falsifications or counterfeiting does rarely require ink analysis beyond optical comparison. This might be due to the fact that ink analysis rarely allows answering the key forensic questions. It is very useful to highlight differences, but the fact that two inks entries cannot be differentiated does not mean that the same pen was used. It may also indicate that a particular ink formulation is very common in the market. One can apply as many techniques as possible, such result will remain particularly

difficult to interpret. Thus, it is also suggested by the authors that advanced methods might not be much more efficient to answer the right questions compared to traditional approaches. As too many resources (in terms of costs and qualified personal) might have to be invested for a slightly increased discriminating power, the investment is generally not worth the effort for small operational laboratories. Only larger national or central laboratories can implement such "emerging" and costly technologies that can then be applied to several other forensic applications. The main impact of such technologies might thus not lie in improved discriminating power, but in versatility (i.e., the possibility to analyse all type of ink composition), reliability (i.e., an objective approach to compare samples taking into account reproducibility of specimen analysis) and non-invasiveness (i.e. the possibility to analyse again the sample). Thus, the highlighted emerging technologies might bring a real impact in practice. Future researches should now attempt, not only to evaluate one method on limited ink samples, but also to compare the different promising approaches using large ink sample sets both, to help select and validate efficient and reliable methodologies, and to support evidence interpretation for judicial purposes. Finally, it is important to remind forensic scientists the importance of blind testing their own methodologies to ensure final validity for implementation in forensic practice [83].

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Chapter 12
End User Commentary on Emerging Approaches in the Analysis of Inks on Questioned Documents

Ana Cristina Almeida Assis

Forensic analysis of inks in a police laboratory aims to clarify for judicial or civil purposes: (a) whether a document has been tampered; (b) the correlation between documents; (c) the correlation between a document and a writing instrument and (d) the correlation between a document and a printing device. To this end, the study, analysis and investigation of the documents' inks are used in order to address the questions formulated by the criminal investigation or by the court, thus contributing to the resolution of crimes associated with the falsification and counterfeiting of documents such as mockery, forgery of documents, fraud or threats. The forensic analysis of documents in this context encompasses not only the analysis of inks but also the characterization, identification and differentiation of various materials such as paper, glues, coatings, laminates, waxes, amongst others, for which it is necessary to use the traditional imaging techniques and advanced analytical methodologies.

Based on the review developed by Weyerman and Techabowornkiat in this Chapter, the present commentary aims to discuss the operational perspective of a police laboratory through examples of real cases, reporting on the different technologies used, presentation the of results obtained and the difficulties encountered in relation to available techniques and emerging technologies.

Inks—Despite the constant technological advances and the innumerable resources for document production, the manual instruments of writing continue to have great forensic relevance and to subsist in daily life; for this reason their correct and effective identification, characterization, differentiation and dating is of great importance. In the Portuguese laboratory of scientific police, 50% of the cases of this kind of analysis include manual instruments of writing.

In the analysis of a Commercial Lease Agreement, where the court's request was to verify that the signatures had been handwritten with the same ink, it was found that the morphology of the inks and their luminescence reactions were identical. Other

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non-destructive analysis techniques such as micro spectrophotometry and micro-Raman were used, and the results were inconclusive in terms of differentiation.

After requesting a destructive analysis at the court, we proceeded to the analysis of the blue ballpoint inks by HPLC with DAD detector, enabling to conclude that the signature 1 was handwritten with an ink writing instrument different from the one used in the signatures 2 and 3. In this case the partial destruction of the document was authorized, however in many cases this is not possible, which is a limitation to forensic analysis. In cases where the inks have similar luminescence and fluorescence reactions and similar spectral behaviour, it is not possible to state whether the ink is the same. Most black inks are a constant challenge in this type of analysis where it is imperative to resort to non-destructive techniques. Many of them have a similar spectral behaviour and only a small percentage of these inks are distinguishable by micro-Raman with a 685 nm laser. What would be desirable is to resort to another technique capable of chemical analysis such as mass spectrometry in order to identify all constituent components of the ink. As mentioned by Weyerman and Techabowornkiat, there are already some studies in this respect that demonstrate the applicability of non-destructive techniques such as DART-MS or EASI-MS in the analysis of inks in documents. In a forensic police laboratory, it will only make sense to acquire these technologies if they have a wider range of forensic applications because of their cost. On the other hand, since destructive techniques can be used, the variety of technologies that can be used increases. Studies conducted by Canada Customs and Revenue Agency, Brazil's Federal Police and National Centre for Forensic Science of University of Central Florida show that ESI/MS analysis is a fast and effective method for analysis of vehicles as well as dyes in the inks.

Inkjet—The technology associated with inkjet printing has evolved very fast over the past 30 years. At present, inkjet printers can be found on the market at reduced prices, producing excellent print quality. This development has promoted a widespread use both domestically and professionally, so that in this police laboratory there has been an increase in the number of crimes involving this type of printing.

In the course of a criminal investigation, searches were carried out on the premises of a suspect on suspected birth certificates forgery. In this case the expert work consisted in first determining the authenticity of the recovered certificates. The certificates were false and were obtained by monochrome inkjet printing, so it became relevant to determine whether these certificates were printed on the printer found at the suspect's home. In the text printed on the certificates there are visible clouds of droplets. These droplet clouds result from the speed of the printing head, the ink flow, and the motion direction of the printing head and the paper. The spatial distribution of these droplet clouds is similar in the certificates found and in the subsequent printing made by the investigators using the suspect printer. The HPTLC technique was used in the analysis of the black inks of the certificates and the black ink in the cartridge of the suspect's EPSON T040 printer, revealing a chemical similarity between them. In this case the use of a non-destructive technique was not a problem since the documents under analysis were false.

HPTLC associated with image analysis obtained with UV light is widely used in this laboratory and by similar laboratories in the discrimination of polychromatic

inkjet prints. As mentioned by Weyerman and Techabowornkiat, this is a fast, economical and fairly discriminating technique.

However not all inkjet inks have the same type of formulation. An example of this is the solid inkjet, which it was used in the falsification of invoices that we received for analysis. By analysing the image, the manner of depositing the ink on the paper and the graphic effect characteristic of a solid ink inkjet printing were determined. The invoices may have been printed on the Xerox 8550/8560MFP (Multi-Function Product)/8570/8870/8700/8900 and WC2424 solid ink print devices. Using Fourier Transform InfraRed Micro Spectroscopy (FTIR MS) analysis, coupled with the use of ATR (Attenuated Total Reflectance) with germanium crystal, the presence of a wax was detected, however this technique did not allow the discrimination of magenta, cyan and yellow inks.

Different Inks—Other printing techniques are used in documents and subsequently subject of forensic analysis. An example was a case that consisted in comparing inks used in the scraping zone of lottery tickets, with the fragments found at a suspect location associated with the alleged perpetrator of an assault. We analysed the various constituents of the suspected material by Optical Microscopy, Fourier Transform Infrared Micro-Spectroscopy (FTIR) and Scanning Electron Microscopy with RX microanalysis (SEM/EDX) to study its morphology and elemental composition. The lottery tickets have been printed by flexography and the material used to cover the scraping area of these tickets has spectral characteristics similar to those of some acrylic polymers and their elemental composition consists mainly of Titanium (Ti), Calcium (Ca) and Aluminium (Al). These compounds are commonly used in ink systems. It was concluded that the material used to cover the scraping area of these tickets and the suspicious fragments were made of solid acrylic rubber paints. However, it was not possible to determine whether these fragments originated from the lottery tickets. It is noted that the majority of academic articles are naturally focused on the most common type of inks, however in the police laboratory there are numerous types of ink for both print and handwriting analysis such as invisible inks or iron-gallium inks.

Toners—Of the numerous cases with this type of material, one stands out in which part of the forensic analysis was undertaken before a search carried out by the criminal investigation, in order to direct this search. The expert evaluation served the purpose to determine which laser printer of the many (dozens) found at the Company premises the questioned documents under investigation had been printed in decreasing order of probability. Using the non-destructive Micro-FTIR with diamond cell technique and using the existing spectral database in this laboratory, 10 printers were identified that employed a toner with characteristics similar to the toner used in the printing of the documents. It is relevant to know that there may be other spectral characteristics that are not present in our database. With this information a printer was seized from this list as being the most likely to have been used. The comparison of printing and toners started always with the use of optical microscopy and analysis of toner deposition morphology on paper. Also, in this search the analytical procedure as recommended by ENFSI was followed, as well by the police laboratories or forensic institutes that are part of this network. In addition to the chemical compatibility of the toner verified

by the spectral analysis, artefacts produced by the printer on the suspect documents were visualized. Together, the techniques used in the analysis of toner presented a fairly good degree of discrimination. It was rarely necessary to resort to SEM/EDX or felt the need for another technique.

Conclusion—The transversal use of optical microscopy and image analysis as source of results in forensic analysis of inks are highlighted in all presented cases. For the interpretation of these imaging results it is important that the forensic examiner has adequate training in questioned documents as well as advanced scientific training in chemistry and analytical chemistry. All methodologies needs to be validated even if it they have already been described in a scientific article. The quality of the forensic response is also measured by the validation of the expertise of a second expert and by participating in collaborative tests conducted with other similar laboratories.

The emerging techniques mentioned in this chapter indicate optical and spectroscopic methods such as Micro-Raman and Micro-IR spectroscopy as being more than just very promising because they are non-destructive and fast. These techniques are already employed by many forensic laboratories for questioned documents. The optical and chemical imaging methods used already provide a very good response potential.

It is thought that the additional technological advancement in this area will be made by the use of MS techniques to increase the discriminating power and perhaps for dating purposes.

Chapter 13
Advances in Analysis of Gunshot Residue

Francesco Saverio Romolo

Abstract Analysis of gunshot residue (GSR), produced by the discharge of a firearm, can assist in the association of a suspect with a crime and in the reconstruction of the sequence of events preceding the crime. The golden standard in the analysis of GSR is scanning electron microscope equipped with a detector for the X-ray emission (SEM/EDX). SEM/EDX allows automatic detection of particles containing heavy elements (such as GSR), imaging of particles detected and chemical analysis by EDX. Toxicological and environmental concerns led ammunition manufacturing towards products not containing Pb and other heavy metals. For SEM/EDX it is difficult to characterise the particles from lead-free ammunition and particles from heavy metal free (HMF) cartridges are impossible to be automatically detected. Possible new alternatives could be electron backscattered diffraction detectors (EBSD), ion beam analysis (IBA), time-of-flight secondary ion mass spectrometry (ToF-SIMS), Raman chemical maps, attenuated total reflectance (ATR) imaging and FTIR spectroscopy or, to a lesser extent due to lack of imaging capabilities, chromatography and mass spectrometry. The evaluation of the time since the last discharge is another interesting forensic problem associated to GSR, needing further research for routine application in casework.

13.1 Introduction

Intentional homicide has involved half a million victims (437,000) across the world in 2012, according to the "Global study on homicide 2013" by UNODC [1], and firearms are the most widely used weapons, accounting for 4 out of every 10 homicides at the global level (Fig. 13.1).

Gunshot residue (GSR) is produced by the ammunition for firearms during its explosion. The inside of a cartridge for firearm in shown in Fig. 13.2. When the trigger of a firearm is pulled, the firing pin strikes the primer cap of the cartridge case. The primer charge explodes and activates the smokeless powder. The subsequent

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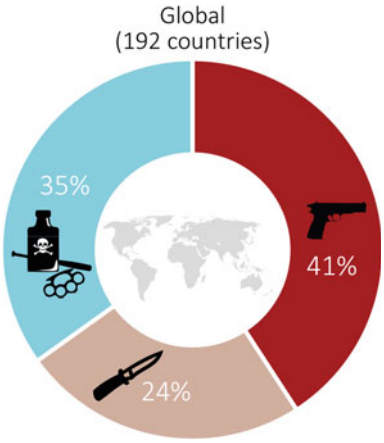

Fig. 13.1 Distribution of causes of death of intentional homicides across the world in 2012. Reprinted from UNODC Global study on homicide 2013. United Nations publication, Sales No. 14.IV.1, Copyright (2014), with permission from UNODC

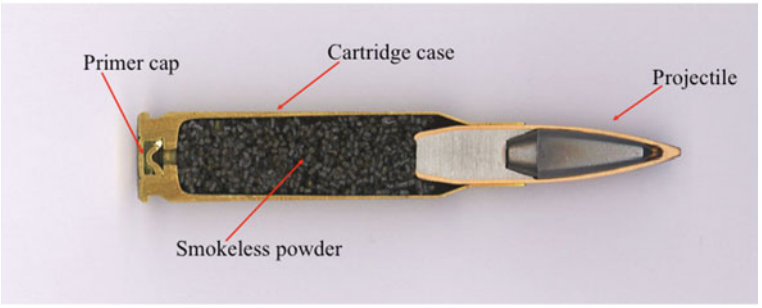

Fig. 13.2 Cross section of a cartridge for firearm. Courtesy of Jean Francois Chevalley

explosion of the smokeless powder produces the hot gases pushing the projectile along the barrel of the firearm [2–4].

The hot gases also push GSR though the muzzle and every opening of the firearm, resulting in GSR deposited around the entry hole produced by the projectile and all the surfaces near the shooting firearm, such as the hands, the head and the clothes of the shooter. A large portion of GSR remains inside the spent cartridge too. The major chemical substance in the smokeless powder is nitrocellulose, producing nitrite ions, nitrate ions and organic nitrated chemical substances after the explosive reaction. This residue was the first GSR analysed for forensic purposes in the so-called "paraffin test" [5]. Molten paraffin wax was used to take a cast of the hand and sprayed with a suitable reagent.

The spot test used in the "paraffin test" gives a positive reaction not only with GSR but also with other oxidisers, resulting in weak evidence of association between the

subject tested positive and the shooting case investigated. On the contrary, the use of spot tests to evaluate the distance between the muzzle and the bullet hole in the target is very useful [6–9]. GSR analysis finds a third successful application in the investigation of firearm-related crimes: the estimation of time since discharge. The main innovative forensic applications based on the analysis of GSR will be described in the following sections.

GSR is the most accepted acronym worldwide and it is used by international organisation such as the Firearms/GSR Working Group of the European Network of Forensic Science Institutes (ENFSI) [10], the American Standard and Testing Materials (ASTM) [11] or the Scientific Working Group for Gunshot Residue (SWGGSR) [12] but it is not the only acronym adopted. Others authors refer to other definitions such as firearms discharge residue (FDR), cartridge discharge residue (CDR), primer discharge residue, potential FDR, or full GSR.

Given that a definition is a "statement of meaning", the acronym GSR is used in the present chapter for consistency reasons. Two more acronyms will be used: OGSR and pGSR. The OGSR is mainly produced by the smokeless powder and contains the organic species originally present in it in addition to many derived transformation products. The pGSR is, on the contrary, mainly the residue of the explosion of the primer mixture and it is composed of microscopic particles resulting from the condensation of vaporised metals. Elements in pGSR can also originate from the projectile, the cartridge case, the smokeless powder and the firearm [13, 14].

13.1.1 Association of GSR to a Shooting Case

The analysis of GSR sampled from the hands, clothes or other parts of the body of a suspect is nowadays essentially based on scanning electron microscopy (SEM) and energy-dispersive X-ray Spectrometry (EDX). The routine sampling approach for collecting GSR particles to be analysed by SEM/EDX is called "tape lifting" and it is carried out using an SEM aluminium sample holder (stub) covered with an adhesive layer. During sampling, the adhesive layer is pressed several times against the surface where pGSR are searched (e.g., hands, hair, face, clothing). After arriving in the lab, the stub is generally carbon coated and analysed by SEM/EDX. This approach is non-destructive and allows the analysis of the pGSR particles on the stub to be repeated.

Forensic interpretation of results begins with examining both morphological and chemical information of particles, typically between 0.5 and 5.0 μm in diameter (Fig. 13.3).

GSR particles from an unknown source found on a specific surface, such as the hands or the clothes of a suspect, are then compared with GSR particles from a known source such as the cartridge case found on the crime scene. Other possible pGSR reference sources are firearms, bullets or bullet holes. The aim of the comparison is to infer about a possible common source of the two samples of pGSR analysed to possibly associate the suspect with the crime [15–17].

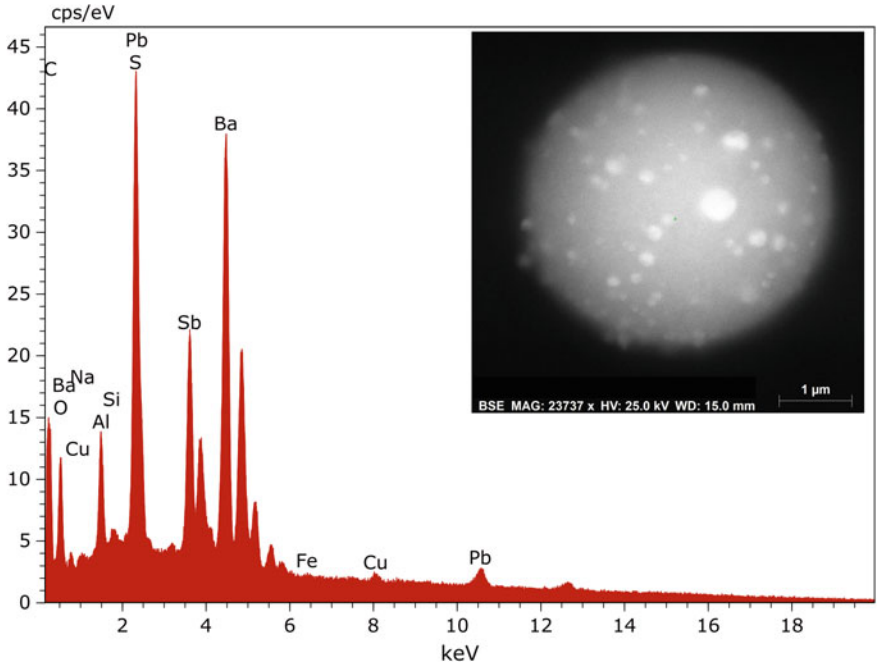

Fig. 13.3 SEM image of backscattered electrons (above right) and EDX spectrum (below) of one GSR particle. Courtesy of Lt.Col. Matteo Donghi, Raggruppamento Carabinieri Investigazioni Scientifiche, Parma, Italy

The procedure followed worldwide for GSR analysis with the aim of associating a suspect to a crime, based on pGSR particle analyses by SEM/EDX, did not change significantly in the last 30 years. The main reason is that the particles in the environment generally do not contain heavy elements ($Z > 20$). For this reason it is possible to make automatic search on stubs and effectively detect particles containing heavy element (such as pGSR) using the backscattered detector (BSD) of a SEM. The particles selected by the automated search are later analysed by EDX and at least one picture of each particle is examined and recorded by the analyst.

In the last three decades technology developments provided more effective automated procedures and forensic research was focused on studying particles produced by the latest lead-free and heavy metal free (HMF) ammunition on the market [18], particles from selected populations [19] or from the environment [20, 21]. There are other interesting subjects such as studies about the formation [22], the fate and behaviour of GSR [23, 24] or approaches for the probabilistic interpretation of analytical results [25–29] but there is no analytical technique expected to substitute SEM/EDX in the near future. This scenario did not discourage research about new analytical tools for GSR and some interesting new techniques showed to be very effective to provide significant forensic information that cannot be obtained by SEM/EDX.

These advances will be described in the two following paragraphs, where pGSR and OGSR are considered separately.

13.2 Advances in PGSR Analysis

When considering pGSR it is necessary to distinguish between bulk analysis and particle analysis. The SEM/EDX approach is a typical case of particle analysis, providing information about the size and the shape of each particle. To explain the bulk analysis approach it is possible to consider that the cast of the hand developed for the "paraffin test" was later analysed by Instrumental Neutron Activation Analysis (INAA). This approach is only able to determine that some elements (e.g., antimony and barium) are present in a sample but it will be impossible to know if the elements detected are in the same particle or were transferred separately, in different moments, from the environment to the hand. This impossibility explains while it is difficult to envisage any bulk technique substituting the particle analysis approach provided by SEM/EDX. About suicide cases, Molina et al. compared a bulk approach by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) and SEM/EDX, concluding that both tests have their benefits and deficiencies [30, 31]. The authors conclude that GSR results cannot be used to differentiate between a suicide and a homicide and in 116 known suicide cases, SEM was only positive in 50% of the cases and only in 18% of cases the pattern of GSR was discernible. The 50% of negative results from person who shot a firearm allows to emphasise that GSR analysis can provide information about the proximity to a discharging firearm or a transfer from a GSR source (e.g. spent cartridge case, firearm, victim) due to contact(s) and cannot be used to determine whether or not a person shot a firearm. ICP-AES also gave only 50% positive results on known suicide cases; it is considerably less expensive and simpler to perform compared to SEM/EDX but the latter gives a clear answer in term of presence or absence of GSR, while the former is not able to produce images of single pGSR particles.

Ion beam analysis (IBA) has imaging capability with suitable spatial resolution to show images of single pGSR particles, providing very interesting results reported in the last few years. In 1982, Sen et al. proposed the use of particle induced X-ray emission (PIXE) for GSR analysis but the size of the beam spot (1 mm^2) at that time did not allow the use in casework [32]. In 1999 Niewohner and Wenz used a focused ion beam (FIB) of gallium with energies of 20–30 keV to cross-section GSR particles produced by four different types of ammunition and to study their internal morphology [33].

In 2009 Bailey et al. published an article about the use of proton beams to analyse pGSR particles, showing that the PIXE intensities were higher than signals obtained by SEM/EDX [34]. In the same year Bailey and Jeynes published another article showing that Rutherford Backscattering Spectroscopy (RBS) can be used to make quantitative analysis of a single pGSR particle [35]. In the following years the IBA approach showed high technology readiness level in providing supplementary infor-

mation in forensic casework by analysing particles already examined by SEM/EDX. In 2013 Romolo et al. used IBA to relocate particles already analysed by SEM-EDX using a typical forensic procedure adopted within the Carabinieri Laboratory in Italy. They showed that the IBA can detect elements at much lower levels than it is possible with current forensic SEM-EDX by PIXE (Fig. 13.4). They also demonstrated that the IBA procedure proposed, thanks to the proton induced gamma emission (PIGE), allows identification of light elements such as B in micron size GSR particles (Fig. 13.5) [36]. This study demonstrates that pictures taken by SEM/EDX of one ore more particles found on a stub and needing further characterisation, allow relocating them later in an IBA facility. IBA analysis of those particles permit the identification of elements not detected by SEM/EDX such as Hg and Zn by PIXE in Fig. 13.4 or B by PIGE in Fig. 13.5. To this author's knowledge IBA results have never been discussed in court. However the interpretation of IBA results based on comparison, as illustrated above, and the presentation in court would be very similar to what is the interpretation and presentation in court of SEM/EDX evidence about pGSR.

Christopher et al. also showed that IBA results can be used to study the grouping behaviour of particles from different makes of ammunition using multivariate analysis in a way not feasible by conventional SEM/EDX [37]. This interesting result needs further development to be used in casework, especially new databases with IBA results from population of pGSR particles from known source. It is interesting to note that the chemical composition of pGSR particles collected from the hands of the shooter and from the bullet hole allow associative evidence to be gathered but pGSR particles from inside the firearm or the cartridges case are more difficult to be associated with samples from other locations [38]. The association between pGSR particle

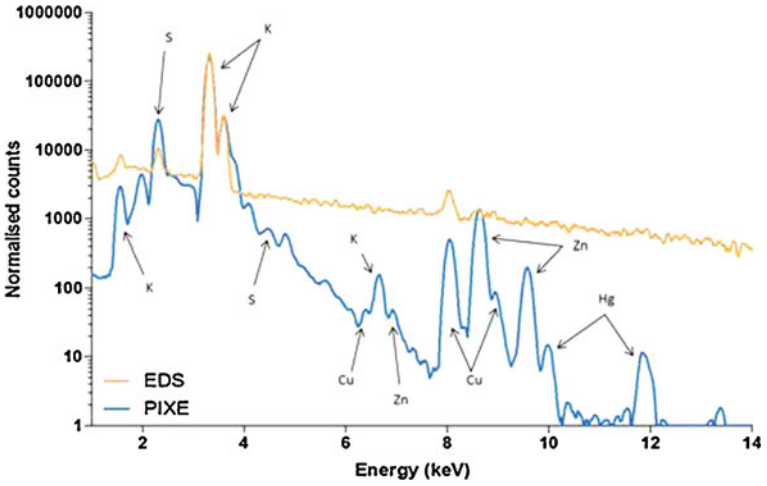

Fig. 13.4 Comparison between SEM-EDX and PIXE signals from the same particle. Reprinted from [36], Copyright (2013), with permission from Elsevier

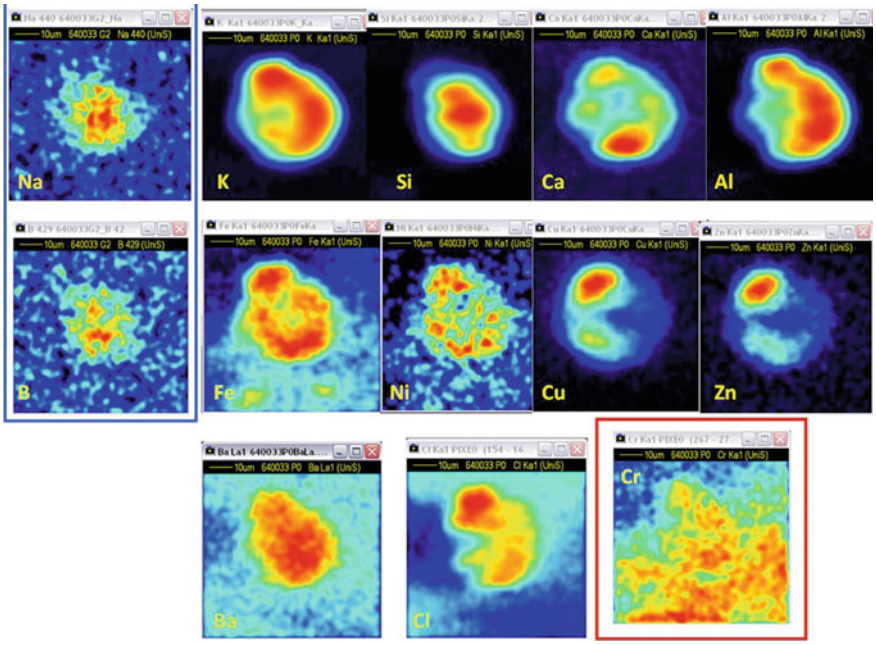

Fig. 13.5 Identification of elements in one particle from CBC Magtech CleanRange using both PIXE and PIGE. In the blue rectangle on the left are gamma-ray maps of Na above and B below. The only elements detected by SEM-EDX are the 5 on the top row (Na in the gamma-ray map, K, Si, Ca and Al in the X-ray maps). In the middle and lower row the elements identified only by IBA (B in the gamma-ray map, Fe, Ni, Cu, Zn, Ba and Cl in the X-ray maps). In the red rectangle the X-ray map of Cr, not related to the particle. Reprinted from [36]. Copyright (2013), with permission from Elsevier

populations analysed by SEM/EDX are based on the number of particles per chemical class. IBA analysis allowed for the first time canonical discriminant function application showing the increase in uncertainty of correlation between two samples of GSR from the same make as number of particles decreases. In real cases, particles on the hands of the shooter are progressively lost and in real cases of homicides the pGSR particles found on hands of suspect is limited to a few [37].

More research producing results about pGSR populations analysed by IBA and suitable databases would provide a more accurate evaluation of the evidential value of pGSR particles in casework.

In 2015 and in 2018 Duarte et al. published results about different ammunitions and their pGSR analysed by IBA, obtaining quantitative results from all constituents of the cartridges and interesting chemical and morphological information about both primers and particles produced after shooting tests [39, 40].

Another feature of pGSR particles is their crystallinity. The first results by Tassa et al. [41] in 1982 were recently developed by Melo et al., who used transmission electron microscopy (TEM) and showed that pGSR particles are partially composed

of crystalline lead oxide domains that are agglomerated into larger particles [42]. Nowadays it is possible to couple SEM/EDX with electron backscattered diffraction detectors (EBSD) and to combine elemental analysis by EDX with crystallographic data from EBSD. In this way Bauer et al. identified the presence of the $TiZn_2O_4$ spinel phase in a particle containing titanium, zinc, and oxygen that originated from lead-free ammunition [43]. It is thus possible to envisage that pGSR particles will be characterised in the future not only by shape and elemental composition but also by crystal structure, considering that some phases, such as the $TiZn_2O_4$ spinel phase, can be produced only in the conditions of extremely high temperature and pressure of the explosion.

For any application in the field in the near future there are two key issues to be considered. The first one is that the routine sampling approach is not expected to change and stubs for "tape lifting" need to be tested before or after SEM/EDX. An interesting example of this situation is that a presumptive lead test, recently proposed to acquire and analyse a sample from a suspect's hands on-site, was conceived to be subsequently analysed by SEM/EDX according to the ASTM standard [44].

The second key issue is the spatial resolution, because pGSR particles on living individual's hands in casework are typically between 0.5 and 5.0 μm in diameter. X-ray fluorescence (XRF) has been considered in the past but showed to be unable of analysing particles smaller than 10 μm [45, 46]. Berendes et al. proposed to use XRF to study the distribution of GSR on different substrates, obtaining successful results with GSR from ammunition without lead, antimony and barium in their primer charges [47].

A very promising approach is time-of-flight secondary ion mass spectrometry (ToF-SIMS), having imaging capabilities of single particles and allowing simultaneous analysis of both inorganic and organic species [48, 49]. Szynkowska et al. used (ToF-SIMS) to visualize and analyse the characteristic GSR particles sampled from different types of surfaces such as wood, metal and plastic by tape lifting. The composition of the pGSR particles was later confirmed by classic SEM/EDX analysis [50].

13.3 Advances in OGSR Analysis

In the last decades, concerns about the toxicity and the impact on the environment of heavy metals such as lead, antimony and barium are bringing more and more HMF ammunition on the market. The particles produced by the explosion of HMF ammunition are more difficult to detect by SEM/EDX, because the intensity of their signals in the image of the backscattered electrons is much lower, compared to particles containing lead, antimony and barium. The increased risk of false negative results by SEM/EDX resulted in a renewed interest for analytical approaches based on chromatography and mass spectrometry. Moreover, the pGSR particles from HMF ammunition have less evidential value. It is also interesting to note that the mass of the primer charge in the cartridge for firearm is much lower than the mass of the smokeless

powder. Thus we should expect that pGSR contributes with a lesser amount to GSR compared to OGSR. The chemical substances more often found in the smokeless powder at significant concentration other than nitrocellulose are nitroglycerine (NG), diphenylamine (DPA), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), methylcentralite (MC) and ethylcentralite (EC) shown in Fig. 13.6. Many authors also consider in their OGSR analytical procedures some degradation products of DPA such as N-nitrosodiphenylamine, 2-nitrodiphenylamine 4-nitrodiphenylamine.

OGSR can be sampled by "tape lifting" using the same stub used for SEM/EDX analysis [51, 52]. Another approach to sample OGSR is "swabbing", commonly used to collect explosives from human skin and smooth, non-porous surfaces. During "swabbing" a substrate such as cotton wetted with a suitable solvent system is rubbed on the surface of interest [53]. A third possible sampling approach for both OGSR and particle pGSR is "vacuum lifting", useful for porous surfaces such as clothes. In "vacuum lifting" particles are trapped on suitable filters using an air pump. The Forensic Science Laboratory of Northern Ireland developed an efficient vacuuming

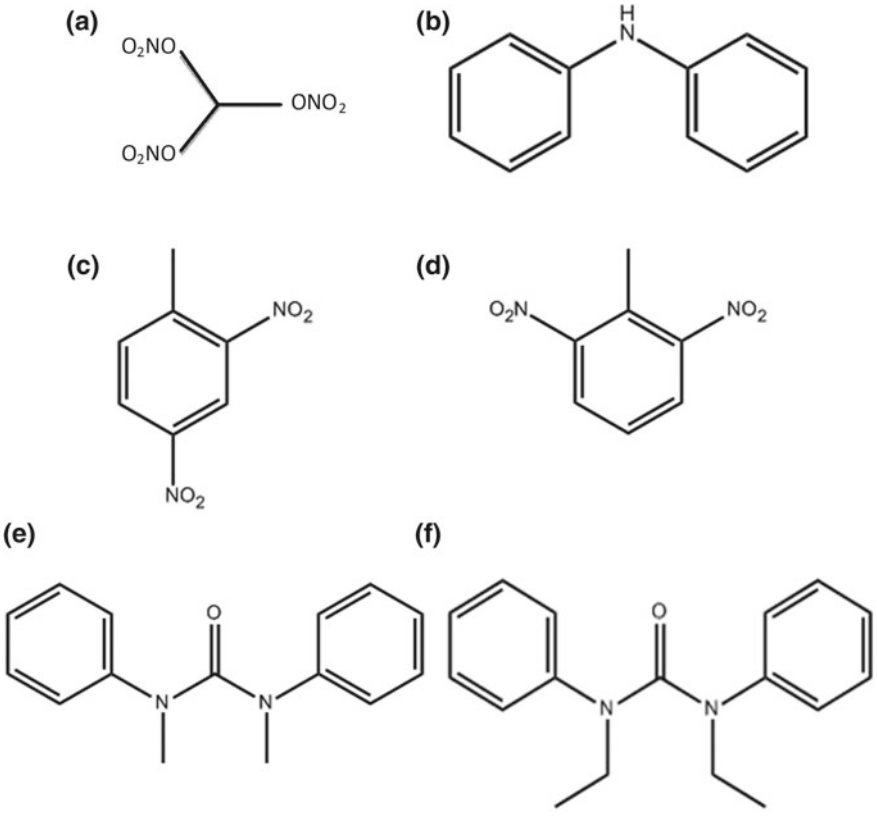

Fig. 13.6 Structures of organic chemical substances in OGSR: nitroglycerine (**a**), diphenylamine (**b**), 2,4-dinitrotoluene (**c**), 2,6-dinitrotoluene (**d**), ethylcentralite (**e**) ethylcentralite (**f**)

system for the recovery of organic and inorganic cartridge discharge residue (CDR) [54, 55].

OGSR often requires a sample preparation after sampling and before analysis. The choice of the sample preparation procedure depends on the type of sample and on the analytical technique to be used. Stubs used for "tape lifting" can be directly analysed by SEM/EDX or microscopy coupled to spectroscopic techniques such as IR or Raman. Swabs can be thermally desorbed before gas chromatography/mass spectrometry (GC/MS) analysis. Stevens et al. used a commercially available thermal separation probe to analyse 27 authentic shooter swabs [56]. Swabs and filters generally need solvent extraction following multi-step processes that include drying and reconstitution in small volumes, resulting in time consuming processes and risk of contamination. Direct extraction of swab by washing it with small portions of solvent results in a large volume of extracts [57]. To reduce the volume of the extract a centrifugal extraction of swabs is possible [58]. It is possible to use a membrane filter during centrifugation to separate the inorganic pGSR particles to be examined by SEM/EDX [55].

Techniques, which can be used to analyse OGSR are capillary electrophoresis (CE), gas chromatography coupled with mass spectrometry (MS), liquid chromatography (LC) coupled to mass spectrometric techniques such as single quad (MS), tandem (MS/MS), high resolution (HRMS), ion mobility spectrometry (IMS), desorption electrospray ionisation (DESI) other MS techniques such as laser ablation (LA) or microscopy coupled to spectroscopic techniques such as IR or Raman.

CE allows the separation of ions in a glass capillary tube filled with a separation buffer based on an electric field application because they generally migrate at different velocities and reach the detector at the end of the capillary at different times. If there are surfactants in the separation buffer at concentration above the critical micelle concentration, micelles are formed in the capillary and they migrate due to the electric field. During their migration, neutral compounds can interact with micelles in a process called micellar electrokinetic chromatography (MEKC), and reach the detector at different times. MEKC was extensively tested to analyse OGSR beginning in 1991, when Northrop et al. published their results of OGSR from spent cartridge cases [59]. Northrop and Mac Crehan also compared many procedures allowing collection, sample preparation and analysis of OGSR by MEKC [60]. Optimised procedures were published in 1998 by Mac Crehan et al. [61] and in 2001 by Northrop [62]. This approach allowed the detection of OGSR on the hands of shooters sampled immediately after shooting, but samples taken one hour after shooting always gave negative results [63]. Reardon and MacCrehan developed a quantitative extraction procedure [64] allowing association of OGSR with the smokeless powder which produced OGSR after explosion [65]. The possibility of analysing OGSR from the hair of shooters was also demonstrated [66]. Morales and Vazquez developed a procedure to detect simultaneously 11 organic and 10 inorganic components of GSR and found that results were in good agreement with those obtained by the bulk approach based on electrothermal atomic absorption spectroscopy [67]. The major issue with MEKC is that a proper and reliable coupling to MS detector is not yet on the market. For this reason the chromatographic procedures, which can take

advantage of the selectivity and sensitivity of MS, are nowadays preferred compared to MEKC.

Stevens et al. found ethyl centralite in 81% of the samples; diphenylamine in 56%, and 2-nitrodiphenylamine in 14% after analysing by GC-MS 27 authentic shooter swabs, who fired between 1 and 5 rounds [56].

Liquid chromatography does not employ high temperatures as GC and is more suitable for thermolabile substances such as NG. Thompson et al. used cotton swabs to sample explosives, water extraction followed by solid phase extraction (SPE) to clean-up and concentrate the sample, and analysed by LC coupled to MS [68]. Tandem mass spectrometry allows for more selective analysis, compared to single quadrupole MS [69, 70]. Several researchers developed a tandem MS approach to analyse stabilizers of smokeless powders present in OGSR [71–73]. DeTata et al. used a HRMS tandem system for OGSR, allowing for superior selectivity and a higher S/N ratio [74].

Ion mobility spectrometry (IMS) is not as selective as the MS approaches described above but it is fast and can be fruitfully if used as a screening test to select positive samples needing further analysis [75].

The DESI ionization technique was introduced in 2004. It enables the in situ analysis of a surface under ambient conditions requiring minimal sample pre-treatment [76, 77]. DESI-MS demonstrated to be a powerful analytical tool. Following this approach Zhao et al. were able to detect EC and another stabilizer called methylcentralite (MC) by DESI-MS and DESI-MS-MS from hands, hair, gloves and various surfaces such as towel, medical gauze, and adsorbent cotton. The method was able to distinguish between 10 shooters and 10 non-shooters by searching MC and EC on their hands [78]. Morelato et al. studied the use of DESI coupled to quadrupole time-of-flight mass spectrometry (QToF) for analysing DPA, EC, and MC. EC was detected on stubs taken after firings, showing potential for the combined analysis of pGSR and OGSR [79, 80].

Abrego et al. used tapes to collect pGSR particles from the hands of shooters and studied the application of LA coupled to ICP-MS to detect seventeen isotopes (15 elements) present in GSR. The system gave a positive detection of one pGSR particle whenever isotopes of Sb, Ba, and Pb were detected. LA-ICP-MS gave positive detection after one shot but the system does not provide morphological information of pGSR particles as offered by SEM/EDX [75]. Moreover the spot size diameter of the laser was of 160 μm diameter, resulting in inadequate resolution for casework [81].

Considering the spectroscopic techniques, Lopez-Lopez et al. studied macroscopic particles from two types of ammunition (one containing DPA and the other EC) by Raman and found that the variation between the spectra of particles within one batch can be rather large [82]. Bueno and Lednev studied the automated acquisition of chemical maps of Raman data from adhesive tape samples with organic and inorganic GSR particles larger than 3.4 μm [83]. They also studied the use of attenuated total reflectance (ATR) imaging and FTIR spectroscopy to find and characterize GSR particles on clothes. They found that both organic and inorganic GSR particles showed characteristic vibrational spectra allowing discrimination between

GSR particles from different types of ammunition, having sizes down to about 5 μm [84, 85]. Bueno and Lednev finally used complementary Raman and FTIR spectra to discriminate GSR particles from 0.38 and 9 mm ammunition. By using statistical analysis, the authors discriminated GSR particles as coming from one or the other firearm/ammunition combination [86].

13.4 Estimation of Time Since Discharge

The analysis of OGSR can be useful to evaluate the time since the last discharge of a firearm and since the explosion of spent cartridges. Andrasko et al. used solid phase micro extraction (SPME) to sample the volatile components of GSR from gun barrels of shotguns and rifles [87, 88], from spent cartridge cases [89] or from pistols and revolvers [90] and analysed them using a GC coupled to a very sensitive and selective detector called thermal energy analyser (TEA), able to detect nitrocompounds, and flame ionisation detector (FID). With the latter detector it was possible to follow the decrease of naphthalene by SPME from firearms and large spent cartridge cases during 2–3 weeks after the discharge of the firearm [89]. Andersson and Andrasko also reported an interesting case of application of the SPME procedure in a suspect murder involving a shotgun [91]. GC-FID and GC-MS were later studied in the following research about time since discharge [92–96], allowing identification of several chemical substances such as naphthalene, 2-methylnaphthalene, 1-methylnaphthalene, acenaphthylene, benzonitrile, phenol, 2-ethyl-1-hexanol, 1,2-dicyanobenzene, diphenylamine.

Recently, Gallidabino et al. proposed an approach based on headspace sorptive extraction (HSSE) [97]. In HSSE a stir bar coated with a layer of polydimethylsiloxane (PDMS) is used to sample from a spent cartridge case, closed in a headspace vial. After sampling, the stir bar is desorbed by thermal desorption (TD) for subsequent gas chromatography/mass spectrometry (GC-MS) analysis (Fig. 13.7).

This new approach allowed the identification of many more chemical substances, mainly due to the larger volume of sorbent phase compared to SPME, allowing for the first time the simultaneous detection of 51 gunshot residue (GSR) volatile organic compounds from fired handgun cartridges. HSSE showed to be more reproducible and effective than SPME. The evolution of volatile OGSR was monitored as a function of time during 31 h with two different types of 0.45 ACP ammunition: Magtech (containing a single-base) and Geco (containing a double-base). The use of compound-to-compound ratios to quantitatively study aging profiles for time since discharge estimation reduced the variability of the aging curves and enlarged the time window (Fig. 13.8). Gallidabino et al. later optimised the HSSE approach, by studying spent cartridges from nine types of ammunition at two different temperatures (20 and 80 °C), and identifying 166 chemical substances in spent cartridge cases, several of which for the first time [98]. Among them, 141 were present in the OGSR of all the cartridges studied. Principal component analysis (PCA) of peak areas due to these substances allows easy discrimination of OGSR produced by dif-

Fig. 13.7 HSSE sampling from a spent cartridge case. Reprinted with permission from [97], Copyright (2014) American Chemical Society

ferent sources. The HSSE approach was later optimised and validated, based on a selection of 29 volatile organic compounds. The resulting process was faster and the use of co-extracted deuterated analogues as internal standards gave improved reproducibility of the measured signals [99]. The optimised HSSE analytical procedure was finally tested on different 9 mm Geco spent cartridges, aged for 1, 3, 5, 7, 24, 31, 48 and 72 h. They were extracted in a oven at 70 °C for 24 h and results were treated with chemometric tools, demonstrating potential to estimate the time since discharge in the up to 48 h of ageing or, at least, to differentiate recently fired from older cartridges (e.g., less than 5 h compared to more than 48 h) [100].

13.5 Conclusion

Forensic analysis of pGSR particles to associate them to a shooting case are based on both morphological and chemical information obtained by SEM/EDX. Based on this information, pGSR particles are classified according to ASTM 2017 as "characteristic particles", "consistent particles" and "commonly associated particles" [11]. To present evidence in court the shape and elemental composition of the pGSR particles of unknown origin (e.g. found on the hands or the clothes of a suspect) are generally compared with pGSR particles from known source, such as the recovered weapon,

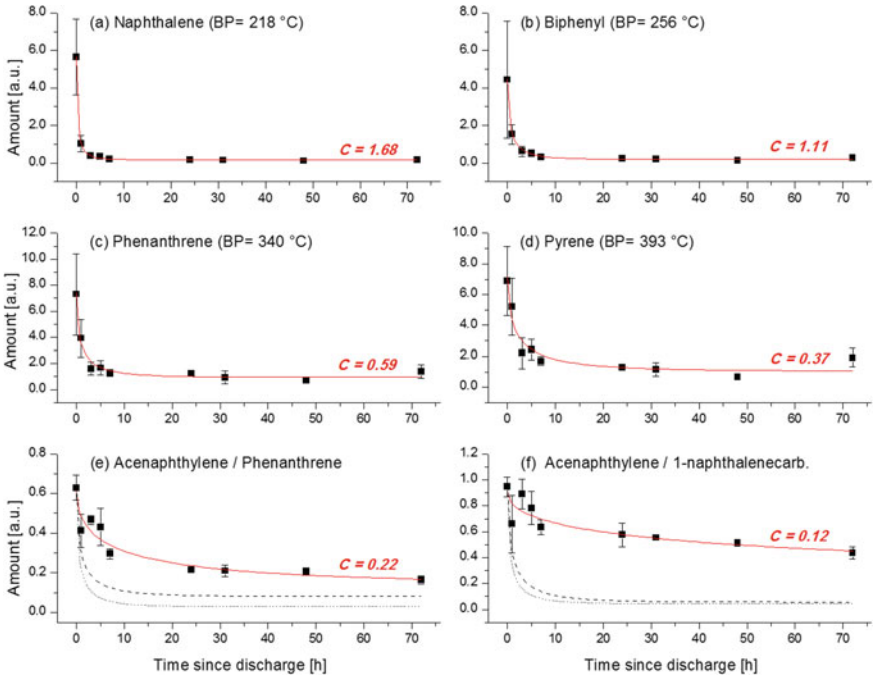

Fig. 13.8 Ageing curves for 4 selected target compounds and 2 compound-to-compound ratios: **a** naphthalene, **b** biphenyl, **c** phenanthrene, **d** pyrene, **e** acenaphthylene/phenanthrene ratio and **f** acenapthtylene/1-naphthalenecarbonitrile ratio. "BP" is the boiling point and "C" is the decrease-rate coefficient. For the compound-to-compound ratios, dashed lines indicate ageing profiles for the respective composing molecules. Reprinted from [100], Copyright (2017), with permission from Elsevier

cartridge cases, or victim-related items following an approach called "case by case" [11, 15, 16, 38].

The interest in the detection and interpretation of organic gunshot residues (OGSR) has produced new analytical approaches, never able to challenge the scanning electron microscopy with energy dispersive X-ray microscopy (SEM/EDX). New analytical approaches can support SEM/EDX by rapidly selecting the most promising items to collect on the crime scene and to analyze or by providing further characterisation of GSR, and their use would be particularly well accepted in casework if the analysis can be carried out on the stub used for "tape lifting". The analytical findings on both pGSR and OGSR in a case would give a stronger association between a suspect and a victim [101]. This expectation about OGSR is not new and a large number of organic GSR identification and characterization methods were extensively reviewed by Meng and Caddy already in 1997 [102].

Because of the prominent position of SEM/EDX as gold standard in GSR analysis, it is difficult to expect a new analytical approach to be introduced in this forensic field without imaging capability with a suitable spatial resolution (pGSR particles

on hands of living persons in casework are typically between 0.5 and 5.0 μm in diameter) and without the possibility to repeat the analysis of stubs, allowing the same presentation in court currently carried out worldwide. The major issue today is that pGSR particles from HMF ammunition cannot be automatically detected by the BSD of a SEM because they mainly contain light elements such as Al (Z = 13), Si (Z = 14), K (Z = 19), Ca (Z = 20).

Another interesting subject for scientific research in the field is the need of suitable databases to provide timely police information (e.g. the manufacturer of the ammunition from the GSR recovered on the victim). There is no standard approach to describe the population of pGSR particles. The major issue is that the populations of particles collected from different locations around the shooter are different, i.e. pGSR particles taken from the hand of the shooter correlate well with pGSR taken from the victim but they do not always correlate well with pGSR from spent cartridge cases [38]. Another issue is the memory effect produced by the ammunition used in previous shooting, leaving a "chemical memory" of elements in the firearm, which are released in following shootings [103]. The consequence is that it is possible that, in the chemical composition of GSR found on a victim, there are elements not present in the ammunition used in the crime.

Forensic interpretation is not limited to shape, size and/or chemical composition of GSR but on studies about their prevalence, transfer and persistence [23, 104–107] and all this information is taken into account for the forensic evaluation of the GSR evidence [17, 108].

The evaluation of the time since the last discharge is a more difficult forensic problem, compared to the association evidence based on GSR. Nowadays there are mainly two approaches: one based on SPME and the other based on HSSE. In both cases they do not have the same maturity of SEM/EDX in GSR analysis and further research is needed for their routine use. As for GSR, further research is not needed only in the collection of analytical information but also in the forensic interpretation the evidence [109].

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Chapter 14
End User Commentary on Advances in Analysis of Gunshot Residue

Sébastien Charles

The field of gunshot residues (GSR) analysis can be divided in two main subfields namely (i) GSR identification on pieces of evidence related to suspects and (ii) GSR identification relating to targets (e.g. victims) mainly for shooting distance determination.

An additional subfield involves reference material that may to some extent establish links between the two above mentioned subfields. Usually most of the inquiries consist of primer-based GSR (pGSR) identification on suspect-related pieces of evidence, determining to generally be the core-business of conventional GSR-units. Target examination can also be performed by these units; however in some laboratories this task is assigned to ballistic-units. Due to the nature of the forensic requests and contingently to local organisation of the laboratories, the articles published in the scientific literature, as well as the research revised in this chapter, may be reviewed by the GSR experts under those different perspectives (suspect/target/reference material).

As mentioned by Romolo in this chapter, Scanning Electron Microscopy coupled to X-Ray Microanalysis (SEM/EDX) has been the method of choice for pGSR identification on suspect-related pieces of evidence since the 1980s. Compared to other techniques, such as Atomic Absorption Spectroscopy, this technique is less prone to issues around source level interpretation, since the particles detected by SEM/EDX and containing lead, barium and antimony are considered as characteristic of GSR. As a consequence, the international norms and guides nowadays only refer to this technique. Since the advent of SEM/EDX automated systems in the 1990s, the development of new types of EDX detectors in the 2000s, and the continuous improvement of spectral deconvolution applications, significant advances have been made with more to come in terms of real time analysis and reduced amount of time spent by the operator performing the review of particles. Because of cost

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reduction and efficiency improvement, this optimization is of major interest for most GSR-units, especially when new SEM/EDX systems are acquired or when considering the quality approach. Unfortunately, only few articles are published in the literature concerning this topic. Raising the awareness of the GSR-community in order to disseminate the studies of procedure optimization and good practice would be a major step forward, allowing small GSR-units to benefit from the experience of bigger GSR-units that have the operational capabilities to perform such optimization studies.

Consequently, due to the lack of publications and result dissemination concerning this specific topic, it may be difficult for an academic (non-end user) to fully report the advances of such technologic improvements in the field of pGSR detection. By contrast, interpretation issues related to pGSR production/persistence/transfer and for which the GSR-expert community also devotes extensive analytical efforts result in the publication of numerous articles.

The link between reference material and the particles produced is also of great interest, according to the case-by-case approach reported in the chapter. This is extensively examined and studied since such issues are often discussed in reports and during trials.

Beside the characteristic of GSR particles for which the origin of shooting incident is well controlled, the indicative particles are, by definition, less characteristic of GSR since such particles could have originated from something else other than a shooting incident. Therefore, caution must be observed: when no characteristic of GSR particles are detected on samples, none or only small-scale interpretation can be undertaken concerning the origin of these indicative particles, sometimes even when reference material is available. As a consequence, the risk of false negative exists, and any technique allowing to confirm the GSR nature of indicative particles could be of great interest. In that respect, and as discussed in the chapter, Ion Beam Analysis (for traces identification) or Electron Backscattered Diffraction Detection (for crystalline characterization) are, in my view, promising techniques for better characterization of particles under investigation.

The GSR scientific community actively prepares itself for the arrival of heavy-metal free (HMF) ammunition in the market since the early 2000s.

The advent of new analytical techniques and the development of different methods for sampling are presented in the chapter. However according to the GSR-expert's point of view, even if this kind of ammunition is nowadays available, the prevalence of such ammunition in casework is still very low, apart, perhaps, for casework involving police forces. The criminal milieu is for sure less concerned about environmental and health impacts of traditional ammunition containing heavy metals. In other words, whilst it is of importance to develop new analytical protocols able to detect GSR produced from HMF ammunition, to date, this is not an issue in most casework examined on a routine basis.

Concerning the subfield of GSR related to target, for which limited developments and studies are reported at this time, the milli-X-Ray Fluorescence (XRF) technique begins to be implemented in some forensic laboratories, as mentioned in the chapter. This is because—unlike what was discussed earlier—the prevalence of HMF ammu-

nition in casework related to distance determination may be higher, since a number of such casework concerns shooting incidents by police forces, which are more frequently using such type of ammunition. In this respect, the milli-XRF technique could be the method of choice by substituting the well-known sodium rhodizonate test when HMF ammunition is used. However, for a large dissemination of such technique in GSR-units, the technology behind this method should be improved, mainly to reduce the instrumental time.

As reviewed in the chapter, the majority of the articles recently published in the field of GSR are related to the detection of organic GSR (OGSR). However, to my knowledge, only a very few number of laboratories have at this moment the operational and technical capabilities to offer OGSR detection in real casework.

In my opinion, considering the identification of GSR on suspect-related pieces of evidence as the main activity of GSR-units, the implementation of OGSR detection in casework suffers from two major limitations, i.e. persistence of residues and sample preservation. For instance, due to several reasons (detailed in the literature), the persistence of OGSR appears to be very low, even lower than the one related to pGSR. Furthermore, once the sampling is performed, the preservation of the samples seems to be of crucial importance, in order to prevent compound degradation. These issues need the development of new sampling kits and procedures in order to ensure a good stability of the target compounds between the time of sample collection on a suspect and the analysis in the laboratory.

Finally, the organisational structure of current GSR-units may be a hindrance to the implementation of such analytical techniques enabling detection of OGSR. Due to organisational and technical reasons, these units are often associated nowadays to material analysis departments. Since OGSR analysis is mainly related to bulk chemistry, current GSR-experts have to change their analytical paradigm, and this modification can be a serious impediment and may take some time to implement such analytical techniques. As compared to the scenario in which the SEM/EDX technique replaced atomic absorption spectroscopy (when the latter was the method of choice for pGSR analysis), I believe only a new technique offering substantial benefits in terms of detection capabilities and assistance to interpretation issues will have the potential to gain the favour of GSR experts.

Besides that, the future of OGSR detection could be the development of on-field tests, with the use of sample kits easy to operate by crime scene investigators.

The studies concerning time after discharge, reported in the literature and reviewed in the chapter, to date, relate to reference material such as cartridge cases, for which the concentration of GSR is very high. However, as mentioned earlier, in most cases the question of interest submitted on a day to day basis is around suspect related pieces of evidence, for which the concentration of GSR is certainly much lower. I believe that even for high contaminated material as cartridge cases, the process behind the handling of such pieces of evidence needs to be optimised, before considering any implementation in casework.

Indeed in many countries, due to logistic and organisational issues, the evidence may be stored for a long period of time before the decision to proceed to the analysis is made. The issue is that the condition of preservation (e.g. in tribunal registries) can

be very poor. As a consequence, these storage conditions (and their impact) should be first investigated and subsequently standardised before pursuing any potential implementation of such analytical service. Nevertheless, time after discharge aspects are already discussed in a pragmatic manner in reports and during trials, due to the potential difference in persistence between pGSR on hands and garments. Indeed, for garments that have not been washed, one can expect a high persistence and accumulation of pGSR in specific area, e.g. pockets. By contrast, based on persistence studies, detecting pGSR on hands may indicate a quite recent contact of an individual with a shooting environment or with a weapon. This is typically the kind of reasoning that may be discussed in reports.

Chapter 15
Advances in the Analysis of Explosives

Francesco Saverio Romolo and Antonio Palucci

Abstract During last decades, the forensic opportunity to detect and identify explosives became more and more important both to protect the safety of citizens and to support the investigations against terrorists and organised crime. The analytical chemistry of explosives has a long tradition of spot test and more traditional approaches, such as chromatography, but has also new tools, such as electro-optical ones, allowing both point detection and remote sensing. In this chapter, four spectroscopic laser based techniques are presented highlighting working principles and capabilities in discriminating explosive compounds at trace level, in field operation, locally or remotely. For each techniques, the detection limits and drawbacks are reported in the application to trace sensing. Such electro-optics tools do not aim to replace the traditional laboratory methods, rather to support them in security applications and in narrowing the area under investigation, reducing the number of samples selected for laboratory analysis. More traditional approaches are then presented and discussed to illustrate the latest development with respect to on-site testing, sampling and analysis by chromatography, electrophoresis and mass spectrometry.

15.1 Introduction

The safety of an individual is a fundamental right as much as life and freedom, according to the Universal Declaration of Human Rights, adopted and proclaimed by the General Assembly of the United Nations on December 10th, 1948 [1]. Thus, detection of explosive materials is a priority task for police forces all over the world. Analytical techniques play a key role to protect airports, land borders, seaports and the whole supply chain or mass transit facilities, such as metro or railways stations, from the threat of bombing. Terrorism and organized crime use improvised explosive devices (IEDs) made out of commercial materials such as triggers, wires, batteries,

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shrapnel, electronic components and one or more explosive charges, everything properly packaged. IEDs are generally concealed and, contrary to military ordnance, the shape of the container does not provide any information about how the device might work. Therefore, the need clearly emerges to detect the suspicious threat with non-contact technologies, before the possible activation of the explosive device.

To protect people's security, analytical chemistry techniques are useful to trigger an alarm due to an explosive charge, whereas physical techniques, such as metal detectors, can spot components of the hidden military ordnance or of the IED (e.g. wires, batteries, shrapnel).

According to statistics from the USA National Counter Terrorism Center (NCTC), more than half of the terrorist attacks are carried out using explosive devices (Table 15.1).

Moreover, the Global Terrorism Database (http://www.start.umd.edu/gtd/) clearly shows the worldwide dimension of the problem (Fig. 15.1), from 1970 to date.

Main targets of terroristic attacks are the metropolitan areas, where they produce a stronger impact on the media. Just to recall the main attackers perpetrated through the use of explosives: Madrid (2004), London (2005), Mumbai (2006), Moscow (2010),

Table 15.1 Distribution of worldwide terroristic attacks according to their types in 2015 (https://www.state.gov/j/ct/rls/crt/2015/257526.htm)

Type of attack	Percentage (%)
Assassination	8
Facility/Infrastructure	8
Hostage taking	8
Armed assault	23
Bombing/Explosion	52

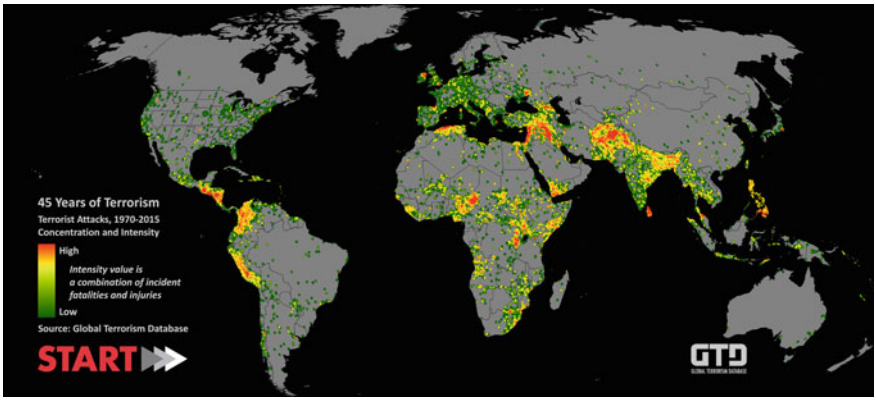

Fig. 15.1 GTD World Map: 45 years of Terrorism displays the concentration and intensity (combining fatalities and injuries) of terrorist attacks that occurred worldwide across 45 years of data [2]

Volgograd (2013), Paris (2015), Brussels (2016). Public transports in large cities are increasingly attractive for terrorists (Fig. 15.2).

Most of the substances classed as chemical explosives generally contain oxygen, nitrogen and oxidizable elements, such as carbon and hydrogen. Chemical explosives have generally high oxygen and/or nitrogen content to react with sufficient rapidity and to maximize the working fluid (i.e., gas) generated in the explosion [3]. According to Meyer et al., explosives, whether compounds or mixtures, are substances, "which are in a metastable state and are capable, for this reason, of undergoing a rapid chemical reaction without the participation of external reactants such as atmospheric oxygen" [4]. The most common functional group in military explosives is NO_2. Explosives can be divided into six groups from a chemical point of view: aromatic nitro-compounds nitroarenes (e.g. dinitrotoluene, trinitrotoluene), nitric esters having the O–NO_2 function (nitroglycerine, nitrocellulose, pentrite), nitramines having the N–NO_2 function (cyclotrimethylene trinitramine, cyclotetramethylene tetranitramine), derivatives of chloric and perchloric acids, and a last group of various compounds capable of producing an explosion, such as fulminates or peroxides [5, 6]. Azides are powerful primary explosives belonging to the sixth group. They are commonly used as initiators (commercial detonators) in civilian and military operations, therefore they could be potentially used by terrorists for IEDs. The peroxide-based explosives such as triacetone triperoxide (TATP) and hexamethylene triperoxide diamine (HMTD), also belonging to the last group, are powerful improvised explosives (IE) which can be easily prepared from ingredients readily available. They have been involved in terrorist attacks mainly in the last twenty years [7–9].

The EU Detection of Explosives and Weapons at Secure Locations expert group has recently highlighted the implementation of high throughput and minimally intrusive detection systems as main requirement for a first level approach in today's explosive detection architectures. This is extremely important in order to have a preselection for slower and more intrusive control activities for an alarm confirmation in infrastructures such as metro and train stations, where it is neither realistic nor appropriate to design and put in place checkpoints like those deployed in the airports [10].

The ultimate goal is to rapidly detect potential threats, and to obtain documentary evidence that, if possible, has also probative value. This is not always possible: according to a prudential approach, a false positive is acceptable in a security scenario but must be avoided when producing scientific evidence in court. When analysing evidence related to a criminal investigation, each positive finding needs to be confirmed before the criminal trial by another analysis, based on orthogonal principles. This approach is needed to avoid sentencing based on false positives but confirmation analysis in forensic laboratories are generally time consuming. An example to illustrate the security scenario is a passenger, who is screened in airports by arch metal detectors and conventional X-ray equipment. After a positive signal, he is further screened by advanced metal, explosive and hazardous substance detectors, and state-of-the-art X-ray equipment. If doubts are not eliminated yet, a complete manual search is finally carried out.

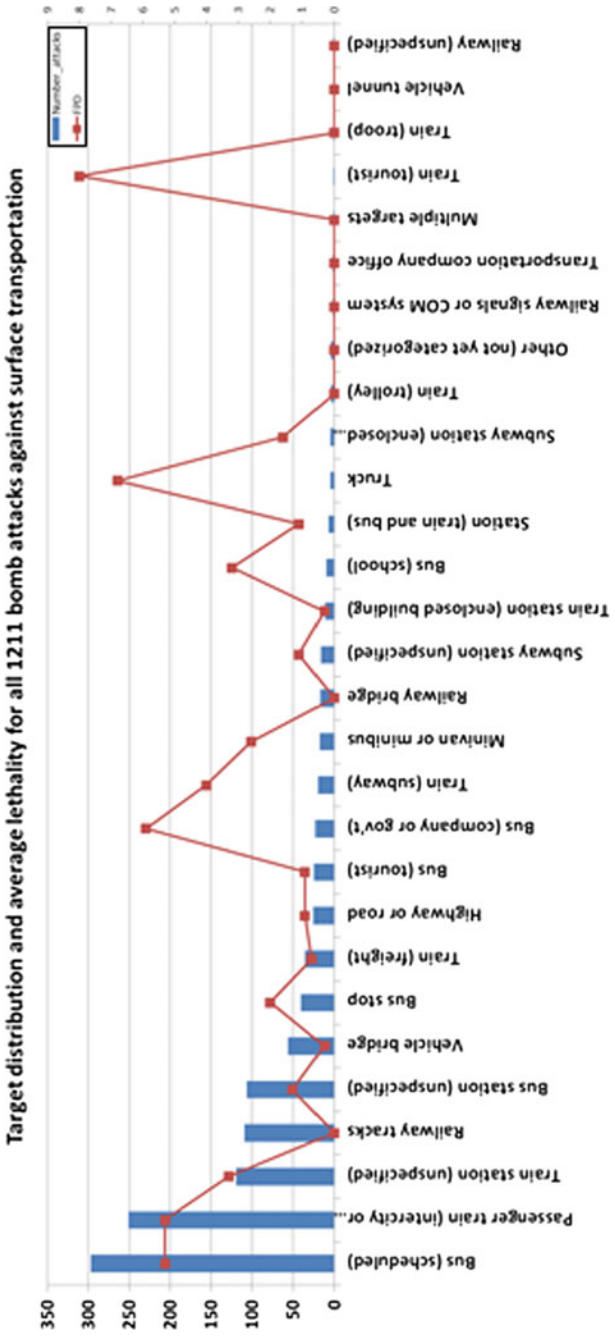

Fig. 15.2 Target distribution and average lethality for all 1211 bomb attacks against surface transportation

Physical techniques (e.g. metal detectors) targeting firearms or IEDs' components such as containers, batteries or shrapnel are not considered in this chapter. With respect to the detection of explosive chemical substances, two possible approaches exist: non-contact bulk analysis and trace analysis.

Good examples of the bulk approach are instrumental neutron activation analysis (INAA) [11] and X-ray diffractometry (XRD) [12, 13].

Neutrons from a suitable source are used in INAA to actively interrogate the materials. Neutrons are not easily shielded, e.g. pass through iron and lead with very little attenuation, so they can interact with the materials of interest, visible or hidden (an example of the latter situation is the explosive hidden in luggage). Neutron interactions produce radiation, including characteristic X-rays, gamma rays, inelastically scattered neutrons, and elastically scattered neutrons [14]. The identification of material is based on detectors acquiring radiation and analysis algorithms. The major drawbacks of INAA are the hazard for nearby personnel to work with neutrons, requiring either significant shielding or large stand-off distances and the potential for materials activation. Moreover, research and development is still necessary in the field to reach the same maturity of robust technologies such as metal detectors and X-ray screening, used for decades all over the world.

In X-ray diffraction an X-ray beam probes samples, the photons are scattered by atoms in a periodic lattice such as a crystal structure. If the incident X-ray is monochromatic, it is possible to record the intensity of X-rays as a function of the angle with the incident beam. If the incident X-ray is polychromatic, the measurement can be performed at a fixed scattering angle with an energy resolved detector. The pattern obtained depends on the atomic planar spacing (d) and on the radiation wavelength (l) according to Bragg's law: $2d \sin q = n\lambda$ (where n is an integer) and can be used to recognize an unknown substance based on comparison with a collection of reference patterns.

If on one hand the bulk approach allows to search for illegal materials inside luggage or boxes, on the other hand, whenever trace contamination is detected on the external surfaces of luggage or boxes, it is necessary to consider that there is an increased probability of a bulk charge inside. Trace detection methods are used on the presumption that: (i) while preparing or delivering a bomb, the bomb carrier themselves or their personal items have become contaminated with a residue of explosive, and (ii) this residue will be detectable at a screening point. This means that the effectiveness of spotting or sampling the trace is as critical as the analytical detection method.

Photonic spectroscopic techniques are commonly used to investigate materials to retrieve information on atomic and molecular energy levels, vibrational modes, molecular geometries, chemical bonds, interactions of molecules, and related processes. Therefore, spectra can be collected at a certain distance, if the trace is correctly detected, and used to effectively analyse components of a sample qualitatively; at least for simple molecules. For other analytical approaches such as chromatography or mass spectrometry, the trace must be sampled and transferred into a device to be analysed.

Finally, it is interesting to refer to the restrictions for passengers on carrying liquids, aerosols and gels (LAGs) aboard, introduced in 2006 following a foiled plot to detonate homemade liquid explosives aimed at blowing up several aircrafts during a flight from London-Heathrow Airport [15]. The restrictions are related to the possibility for criminals of using concentrated hydrogen peroxide (HP) to prepare explosives. Based on this possibility, a new approach to locate illegal manufacturing sites of IE has been proposed [16, 17].

Criminals manufacturing improvised explosives (IE) use chemical substances called precursors. These compounds are released in the air and in the waste water during IE illegal production, allowing bomb factories' localization by tracking sources of precursors based on air or wastewater analysis [18]. A key role for such a task is played by the system interpreting the analytical results to trigger an alarm when at least two ingredients, used in the same recipe to produce an IE, are detected in the same area at the same time [19]. Forensic science should not just be considered as the discipline of evidence to be searched after a crime but in the broadest possible meaning, including the capability to provide information before the crime [20].

During criminal investigations, the two main types of forensic problems are bulk analysis and trace analysis of residues. General comprehensive schemes for the analysis of post-explosion residues where first described in the 1970s. They can include the team approach for processing bomb-scene, visual examination of debris, sample preparation and analysis [21]. Analysis of traces on suspects or on their belongings are carried out with the same analytical techniques used for post-explosion residues but with different sampling approaches. The work of experts after the bombings occurred in Bali on 12th October, 2002 is a good example of the importance of having timely, albeit tentative analytical information at the crime scene [22]. Sensors for fast screening of post-blast evidences are very important, reducing the number of evidences sent to the forensic laboratory and the time of data acquisition, and increasing the information provided by the evidences left by the explosion during the early stages of the investigations. For this reason portability of analytical devices is a key feature for their effective use.

In this chapter the most interesting advances are grouped according to techniques. The first two techniques described are stand-off, allowing remote detection of explosive traces from a certain distance (detection up to 100 m). The other techniques require the sample to be inserted into a machine to be analysed. The stand-off approach is particularly interesting in police activities, allowing analysis from hidden positions of people clothes, doors knobs, cars handles. The different techniques will be presented underlining the main advantages and benefits for end users. It is important to stress that there is no "magic wand" and each security and investigative scenario needs a multi-techniques fitness for purpose approach. In general end users' requests for new tools are that they need to be easy to operate, possibly handheld, possibly stand-off, and that they must have high selectivity and high sensitivity, resulting in a high rate of true positive results and a low false alarm rate (FAR).

15.2 Laser Induced Breakdown Spectroscopy (Stand-Off)

One of the techniques for ultra-rapid, in situ identification of materials is the laser-induced breakdown spectroscopy (LIBS) [23], as it does not require any sample preparation. LIBS analyses require no contact, making stand-off detection possible up to 130 m [24]. LIBS is based on plasma generation by an intense laser pulse, which leads to atomization and ionization of the sample material. Spectral emission from the excited species in plasma state is used for the detection, identification and eventual quantification of the sample composition.

Explosives are chemical substances, containing carbon, hydrogen, and oxygen, and nitrogen is often present. Commonly, explosives are rich in N and O, and poor in H and C. LIBS spectra from energetic materials normally contain atomic lines from these four elements and molecular bands of CN and C_2 [25, 26]. Molecular emission can be also attributed both to the native C=C and C–N bonds [27, 28] and to recombination in plasma [29]. Rapid LIBS detection of energetic materials is normally performed in air, so the interference from air components on the spectra must be considered. Classification of organic compounds by LIBS can be performed by comparison between the sample spectra and the previously established library, or by comparing line intensity ratios from H, C, N and O [24, 25].

An example of LIBS spectra from nitrocompounds deposited on black plastic material is reported in Fig. 15.3. For each spectrum at least 8 different points have been analysed on the sample surface. All the measurements (in light gray), together with the average spectrum (black line) are reported in each panel. The acquired spectra show the presence of emission peaks associated with C (247.8 nm), Mg (279.5 and 280.3 nm), Ca (393.3, 396.8 nm), Na (589 nm), Li (610.35, 670.78 nm), H (656.3 nm), N (742.4, 744.3, 821.6, 824.2 nm) and O (777.2, 777.4, 868.1 nm) [30, 31]. The CN UV system (385–388 nm), which is characteristic of organic compounds, and the vibrational bands of the carbon molecule C_2 Swan system (415–421 nm) are also clearly visible in the spectra.

Different commercial sensors are available on the market [32, 33], but the complexity falls in data analysis as clearly demonstrated from Fig. 15.3, with multi-elemental lines. The use of dispersing optical elements as high resolution monochromators require a very detailed spectral calibration by using calibrated lamps or standard targets, and finally validated with calibrated samples similar to the targets. Furthermore, a procedure for explosive extrapolated features recognition is based on appropriately developed algorithms, or on chemometrics. Selectivity is good and sensitivity is very high (less than nanogram).

With the LIBS technique, the laser energy is focused in a very small area and reaches very high intensity, thus equipment must be used according to international eye-safe regulation IEC 60825, limiting the laser exposure and defining the MPE (Maximum Permissible Energy). This is a drawback for the LIBS technique to be not applicable in public places.

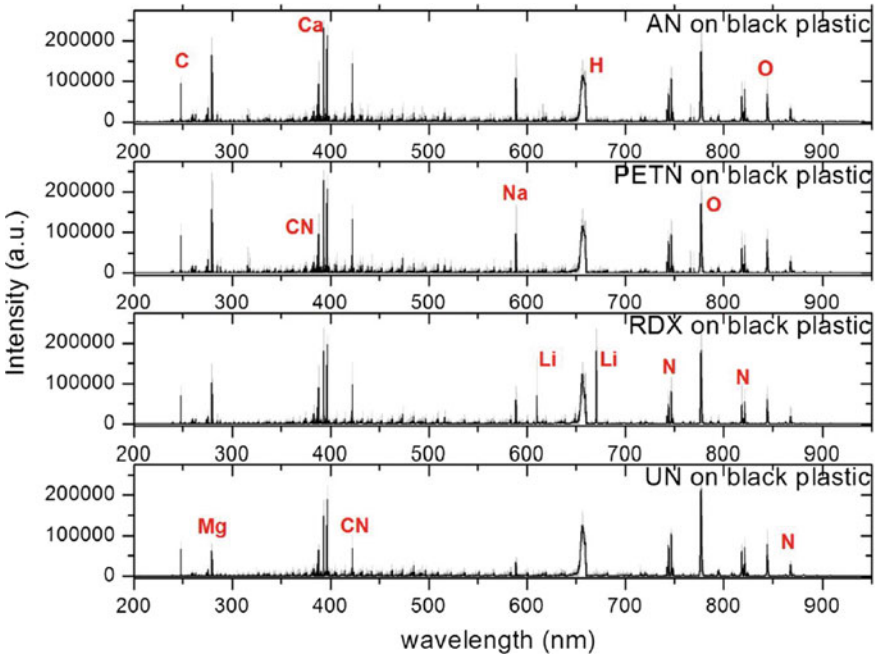

Fig. 15.3 Representative LIBS spectra of HEM samples, ammonium nitrate, pentaerythritol tetranitrate, cyclotrimethylenetrinitramine, urea nitrate on black plastic in fingerprint concentration. The most intense atomic and molecular emissions are also given

15.3 Raman (Stand-Off)

Raman based spectroscopy [34] has gained consensus as a potential tool for detecting trace explosives because of its high discrimination capabilities and extremely appealing application in remote sensing, being based on molecular detection where each substance gives rise to peculiar spectra [35–37].

The majority of explosive chemical substances contain nitrate ($–ONO_2$) and nitro ($–NO_2$) functional groups. Raman spectra of nitro functional group are usually identified through the symmetric (~1250–1375 cm^{-1}) and anti-symmetric (1487–1630 cm^{-1}) $–NO_2$ stretching bands. The strongest (1356 cm^{-1}) band derives from $–NO_2$ symmetric stretching coupled [36].

Raman spectroscopy technique has been implemented in the context of STANd-off Detection of Explosives (STANDEX) programme, a research project under the NATO Science for Peace program devoted to develop an explosive warning system designed to work in an existing mass transit infrastructure [38]. The new remote analytical system developed in STANDEX was called RADEX (Raman Detection of Explosives) and was successfully operated in the BCT (Big City Trial) test in June 2013 in the Metro Station Francois Mitterrand Paris [39].

The RADEX apparatus has the following performance characteristics:

- proximal detection (6–7 m)
- real time investigation
- operation in respect to MPE
- remotely controlled
- integrated in a sensor's network
- preliminary algorithms for data retrieval
- tested in a real environment

In the layout of the control software (Fig. 15.4), the user can view the following information: streaming video centred on the point to analyse (1), the collected Raman spectrum (2), the logging data and messages to the operator (3), the small field of view camera snapshot of the analysed area (4) and the real time result of the analysis in form of a traffic light showing the alarm level (5).

Fig. 15.4 Layout of the RADEX acquisition software during remote analysis of the red spot in the blue square shown in the above two pictures: streaming video centred on the point to analyse (1); the acquired Raman spectrum (2); the logging data and messages to the operator (3); the small field of view camera snapshot of the analyzed area (4); and the real time result of the analysis in form of a traffic light showing the alarm level (5)

The video camera adopted is a special device allowing to record the position of a red laser beam for pointing the invisible, overlapped laser spot.

Different algorithms for data analysis have been developed with the main aim to detect in real time the presence of suspicious substances and designed to remove uncorrelated noise and subtract the fluorescence contribution. In this respect, a discrete wavelet routine was introduced to recover the spectral signature and assign to the corresponding chemical substance (Fig. 15.5).

RADEX was calibrated with reference samples of explosives realized at R.I.S. Carabinieri laboratory (Rome, Italy) and in the Fraunhofer Institute for Chemical Technology (Pfinztal, Germany) [40]. Several microscope slides, covered with different fabrics were prepared, depositing each explosive in solution (trinitrotoluene, urea nitrate, pentaerythritol tetranitrate and ammonium nitrate) on a squared area of 1×1 cm^2 to have a surface density between 100 and 800 μg/cm^2. The procedure allowed, after the solvent evaporation, to obtain micro-particles of explosives homogeneously distributed on the surface.

Implementation of statistical analysis through PCA allowed improving the discrimination ability of the data analysis algorithm, allowing identification of explosives on different fabrics (blue polyamide, black polyester, brown leather). As an example, in Fig. 15.6 the score plots of the three principal components are shown, which explain about 80% of the spectral variance of the three datasets. Observing the PC score plots, the selected samples seem to cluster satisfactorily into distinct groups except for the latter case, in which the trinitrotoluene (TNT) cluster is superimposed to the clean, brown leather cluster (green and red dots) and the pentaerythritol tetranitrate (PETN) cluster is barely distinguishable from the previous two. The main difference in this case is that the first principal component accounts for more than 64% of the total variance of the spectrum, while the other components give a relatively low contribution; in the previous case the second principal component accounts for a larger contribution (about 13%), allowing for a better clustering in the plane delimited by the PC1 and PC2 axes. The third component is relatively irrelevant in this analysis, accounting for almost the same, residual variance in all the PC scores.

In order to derive the detection capabilities of the apparatus, a data evaluation program was developed and tested on the experimental data to explore the potentialities for an automatic recognition of explosives. The program is based on the comparison between the intensity of the stronger band of each explosives versus a reference threshold. Its performances have been evaluated via ROC curves [40].

Receiver operating characteristic (ROC) curves were used to discuss and quantify the sensitivity and the selectivity of the proposed recognition procedure. In Fig. 15.7, ROC curves are reported in terms of selectivity, or true positive rate (TPR), i.e. the TP/(TP + FN) ratio, as a function of (1—specificity) or false positive rate (FPR), i.e. FP/(TN + FP) ratio. About 120 spectra of fingerprints of explosives were processed, together with 120 spectra of the clean leather, changing each time the threshold values from 0 to 0.2, in step of 0.01 and getting the corresponding TPRs and FPRs, to build the ROC curves for each compound. The qualifying parameter considered for the presented binary classifier is the Area Under the ROC Curve (AUC), reported in the graph legend for each explosive. The higher the AUC the better is the classifier.

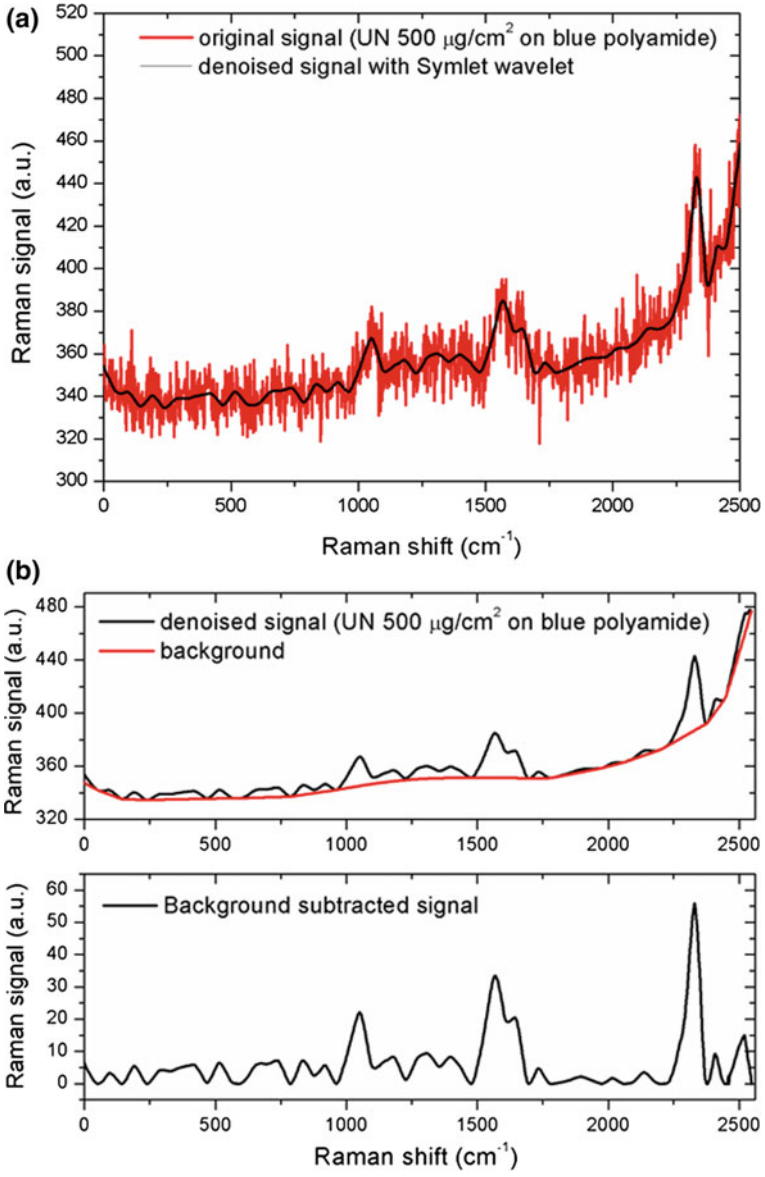

Fig. 15.5 Raw Raman spectrum of a single shot eye-safe UV laser (redline) compared to the de-noised spectrum (blackline) obtained with a CWT Daubechies least symmetric wavelet (Symletwavelet). **b** (Top) Plot of the denoised Raman signal (blackline) and fluorescence background (redline) after the application of the subtraction algorithm. (Bottom) Plot of the background subtracted signal

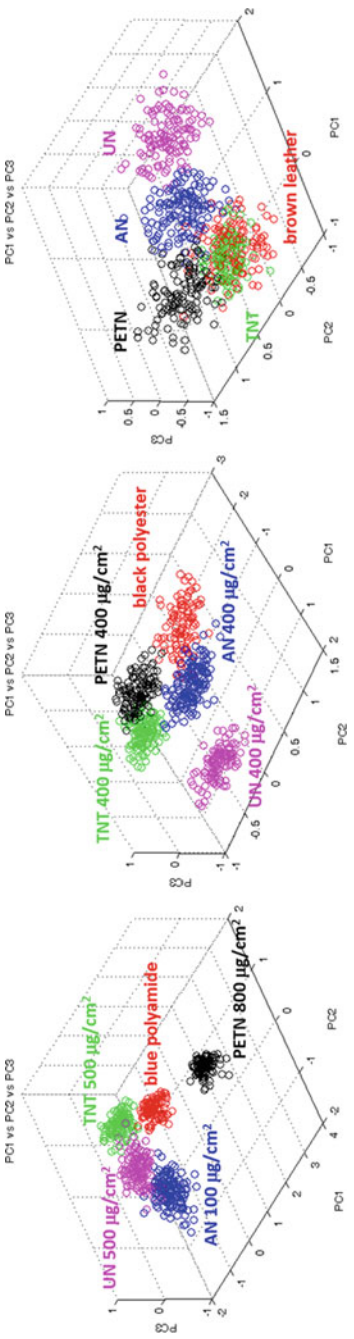

Fig. 15.6 PCA score plots showing discrimination between explosives compounds and clean substrates of different fabrics or tissues. The superficial concentration of each explosive is reported in the first two graphs while the third figure show discrimination of explosives deposited on brown leather simulating a single fingerprint

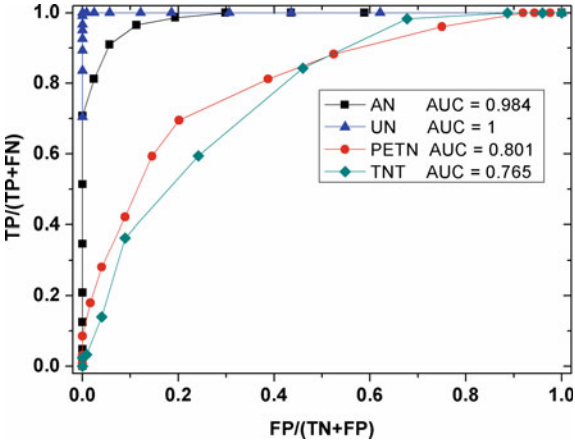

Fig. 15.7 ROC curves for the examined explosives deposited on brown leather in fingerprint concentration. In the legend are reported the corresponding area under the curve

To our knowledge, the RADEX device is at the highest sensitivity nowadays achievable in the field of eye-safe, Raman devices for proximal detection. The actual performances achieved in a proximal stand-off detection of trace of explosives on fabrics [39] are target LoD 100–1000 $\mu g/cm^2$ at a distance of about 6–7 m.

Raman spectroscopy is useful not only to analyse explosives but also precursors of explosives. Hydrogen peroxide in aqueous solutions has been detected through containers or packaging using Raman spectroscopy [41–43]. Ramírez-Cedeño et al. [44] reported analyses of H_2O_2 concealed in mixtures with alcoholic beverages, such as whiskey, rum, and tequila via Raman. Pettersson et al. demonstrated the stand-off detection capability of Raman with 5% H_2O_2 solutions in water through coloured glasses and PET at 55 m [45]. Stewart et al. proposed a handheld Raman spectrometer to determine H_2O_2 concentrations in liquids found at suspected IE manufacturing sites [46].

15.4 Surface Enhanced Raman Spectroscopy

The Surface Enhanced Raman Spectroscopy (SERS) technique overcomes the shortcomings of Raman spectroscopy and requires little or no sample preparation.

SERS enhancement mechanism occurs when a molecule is on or near a metallic nano-scale roughened substrate, and the mechanism appears to derive from two separate contributions: (1) an electromagnetic enhancement and (2) a chemical enhancement.

The former comes from the electromagnetic interaction of light with metals, which produces large amplifications of the laser field through excitations generally known as plasmon resonances [47–49]; the latter corresponds to any modification of the Raman polarizability tensor upon adsorption of the molecule onto the metal surface [48, 50]. This technique emerges as one of the most interesting and prompt tools for

label-free high sensitive detection of different molecules, chemical, biological, toxic or explosive compounds [51].

The experimental set-up [52] consists on an integrated Raman system (BWTEK inc., i-Raman), operated in the wavelength range 789–1048 nm corresponding to Raman shifts of 75–3200 cm^{-1} (resolution 3 cm^{-1}). The Raman system is equipped with a micropositioning system for fine xyz adjustments and a video camera for sampling viewing. Depending on the mounted microscope objectives, the laser has a diameter of 180, 90 or 50 mm.

Commercially available solutions have been used for the experimental test namely pentaerythritol tetranitrate (PETN) in methanol (Superchrom, 1, 100 mg/ml), ethylene glycol dinitrate (EGDN) in methanol (Sigma, 1, 100 mg/ml), cyclotrimethylenetrinitramine (RDX) in methanol/acetonitrile (Superchrom, 1, 100 mg/ml) and trinitrotoluene (TNT) in methanol/acetonitrile (Sigma, 1, 100 mg/ml).

The preparation protocol consists in the deposition of a controlled volume of solution onto the substrate and the successive evaporation. Raman measurements are carried across the deposited patch, clearly visible under the optical microscope matched with the spectrometer. In our procedure, substrates fabricated by depositing a gold layer on a silicon surface with an ordered nanostructure produced by microlithography (Klarite, Renishaw diagnostics) was employed without any pretreatment. Typical case is a drop of 0.1 μl at a concentration of 100 μg/ml, expanding over an area of 5 mm^2, corresponds to about 13 pg of analyte detected in the laser beam area of 6.4×10^{-3} mm^2. In the measurement protocol, after the deposition inside the cell, the sample is illuminated with the laser emission for few seconds.

The SERS spectra of the explosives PETN, RDX, TNT, EGDN in the spectral region 250–2500 cm^{-1} are shown in Fig. 15.8; Table 15.2 lists the Raman wavenumbers and vibrational assignments of the principal characteristic bands of these explosives tested. Explosive compounds were detected and identified at trace level quantities, as low as tens of pg [53].

Nowadays, most of the SERS research is focused on using nanoscale junctions between metallic particles such as colloids [57] or lithographic arrays of nanostructures [58]. In particular, the nanostructures arrays have the peculiar property to show strong, sharp and tunable plasmon. Thanks to this feature they are efficient only to detect specific molecular structures.

Hakonen et al. have reviewed SERS Raman as a tools for explosive trace detection, finding it "potentially the major in-field technique", but also extending its potentiality to the chemical warfare agents detection. They have collected properties of a selection of handheld Raman spectrometers and reported suitable substrates and sensitivity data [59].

15.5 Laser PhotoAcustic Spectroscopy

Photoacoustic (PA) effect, occurring even in solid compounds, has been known since 1880, when Bell [60] observed the audible sound produced by chopped sunlight

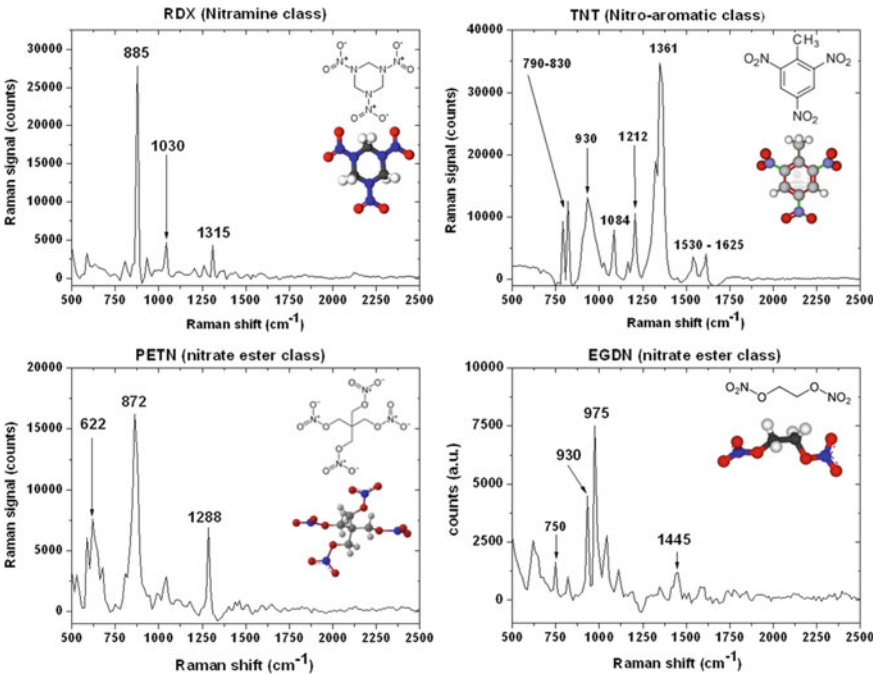

Fig. 15.8 SERS spectra of PETN, RDX, TNT, EGDN. The mass probed by laser is about 200 pg for each substance

impinging an optically absorbing material. A new impulse to this technique arrived with the application of tuneable laser devices as the CO_2 laser in 1968, due to the high beam quality profile, narrow spectral bandwidth and high power emission of laser with respect to the conventional light sources for spectroscopy. The latest sources are ideal to push the sensitivity of PA detection in the ppb (parts per billion) range or even below having high power emitted (order of watts) and tunability on strong fundamental vibrational transition lines.

A classic experimental set-up in LPAS (Laser PhotoAcustic Spectroscopy) technique is shown in Fig. 15.10, where the laser source is frequency stabilized in the single emission IR mode along the 9–11 μm wavelength range. The signal collected by a high sensitive microphone is synchronized by a lock-in amplifier.

In the LPAS technique, the sample is located in a chamber without time consuming sample preparation. The photoacoustic chamber can host small quantities of liquid or solid sample (in the order of 1 mg). Vapour gases can be profitable detected too in case of closed chamber, modified for the purpose. In both cases, after the sample addition, the measurement cycle starts with the scanning of the sample at the different wavelengths emitted by the laser source. In case of a CO_2 laser, more than 60 emission lines can be sent to the sample in sequences and the digital response to the excitation is stored and successively analysed.

Table 15.2 Spectral identification of main bands adopted in the SERS identification

Explosive	Wavenumber (cm^{-1})	References
PETN	1290, [ν_s (NO_2)] 871, [ν_s (O–N)]	[53]
EGDN	1600 [ν_{as} (NO_2)] 979 [C–O stretching] 941 [C–H_2 vibration] 756 [O–NO_2 umbrella]	[54]
TNT	1540, [ν_{as} (NO_2)] 1360, [ν_s (NO_2)] 1212 [C–H breathing] 790–822, [(NO_2) scissor] 796–827 [C–H bending]	[55]
νRDX	1584, [ν_{as} (NO_2)] 1361, [ν_s (NO_2)] 1318, [C–H_2 wagging] 1260, [C–H_2 scissoring] 887 [C–N–C ring] 592 [O–C–O stretching]	[56]

Before undertaking the final identification by PCA, the collected experimental spectra are normalized and background is subtracted. Already the first two components can be sufficient to describe the data set, as shown in Fig. 15.9, thus allowing to unambiguously to separate four groups, each corresponding to an explosive component

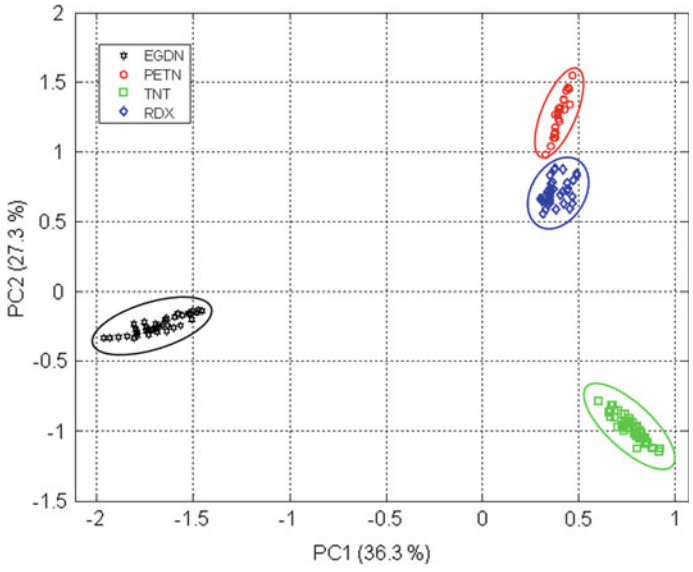

Fig. 15.9 PCA 2D score plot of PC1 versus PC2 for the SERS and Raman spectra dataset. Numbers in parentheses on each axis represent the percentage variance that each principal component accounts for

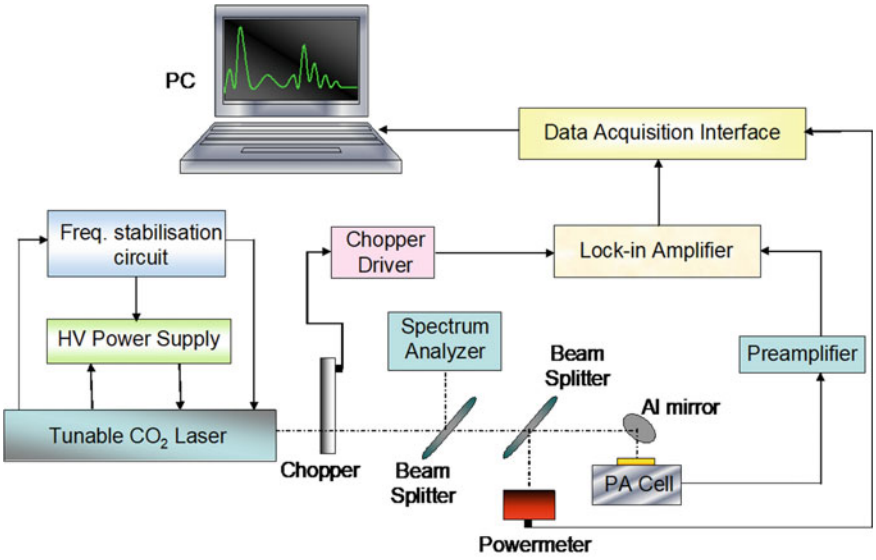

Fig. 15.10 Block diagram of the PA apparatus

An internal database obtained with standard components [31] is mandatory to avoid overlap with other constituents. Robust statistical analysis is requested to extract the features needed to identify the targeting materials. This technique is recently adopting new performing lasers thus allowing compacting the sensor.

Giubileo et al. [61] reported the detection of photo-acoustic spectroscopic characterization and identification of some classical explosives in solid phase (2,4-dinitrotoluene; 2,6-dinitrotoluene; cyclotetramethylene tetranitramine; TATP; PETN), at CO_2 laser wavelengths (9.2–10.8 μm). As it can be observed in Fig. 15.11, the three samples show complex absorption spectra. PCA, therefore, is needed to allow discrimination among the ten samples investigated (Fig. 15.12).

LPAS is a versatile technique thus allowing implementation in different branches of monitoring as in food adulteration [62] or plants affected by pathogens [63].

15.6 Sampling, On-Site Testing and Sample Preparation

There are several scenarios requiring the analysis of explosives. For example, biological samples from personnel working in manufacturing plants are analysed to detect and quantitate traces of explosives and their metabolic products. Most explosives are toxic and their production and disposal pose a serious contamination problem. Moreover, some organic nitrates are used both as explosives and as drugs. Extraction procedures and analytical methods for the measurement of explosives and their degradation products in soil and water are becoming subjects of increasing interest

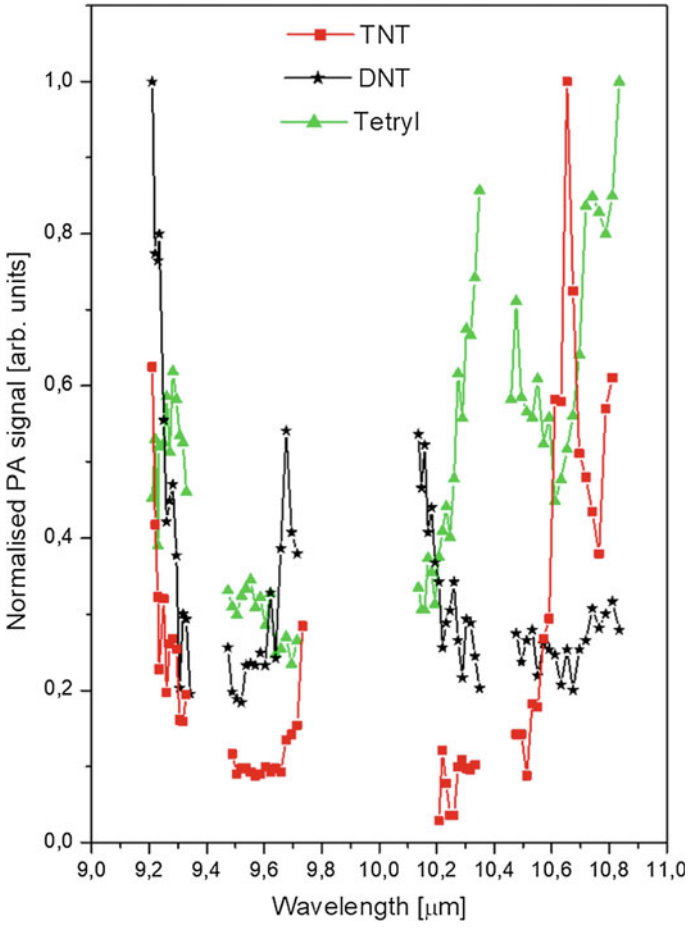

Fig. 15.11 Normalized LPAS spectra measured on dinitrotoluene, trinitrotoluene and tetryl samples

in analytical chemistry too. The closure of former ammunition plants and military facilities requires accurate characterization of the extent of soil and groundwater contamination.

The first studies about the forensic analysis of explosive traces were published in the 1960s [64]. This subject can be sub-divided into two topics: post-explosion residues and traces on suspects or on their belongings. General comprehensive schemes for the analysis of post-explosion residues where first described in 1970s. They can include the team approach for processing bomb-scene, visual examination of debris, sample preparation and analysis [21]. Yinon and Zitrin published the most important reference works in the field [64–66].

When looking for traces, the choice of a correct sampling method is critical. If the traces are searched in the soil of a crater where an explosion occurred, the

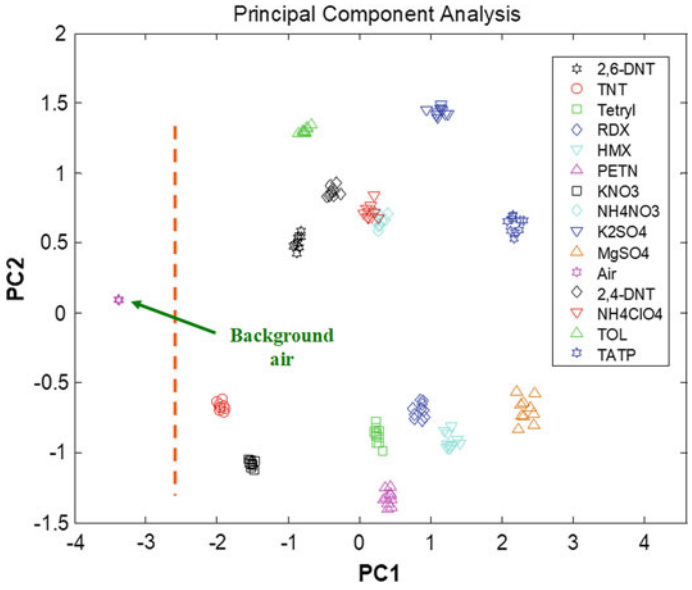

Fig. 15.12 2D scatter plot of the photoacoustic measurements

material from the crater can be removed, transported to the lab and treated with solvents. When the traces are searched on surfaces, the sampling method chosen depends on the nature of the material to be examined (skin surfaces, clothes, etc.). For smooth surfaces a piece of material can be soaked in a solvent and rubbed over the surface being examined. This procedure, called *swabbing*, is the method of choice for skin, work surface, floors and smooth fabrics such as leather or plastic (Fig. 13). On fabrics with an open weave, such as tweed or loosely knitted fabrics, *vacuum lifting* is generally preferred. Traces are trapped on a filter using a flexible tube and a vacuum pump. Particles can be also collected by adhesive *tape lifting* on both smooth surfaces and clothes.

During *swabbing* a substrate such as cotton, wetted with a suitable solvent system, is rubbed on the surface of interest [67, 68]. Different materials for swabbing suspects' hands were tested: cotton-wool, synthetic wool, filter paper, non-woven cotton cloth, acrilan. An ideal swabbing system should efficiently remove the residual explosive from the hands with as little co-extracted interferents as possible. Also, the explosive should remain stable in the swabbing solvent. In water solution, for example, explosives can be degraded by hydrolysis and bacterial activity. The boiling point of the solvent is another important factor: Douse [69] used ethyl ether to avoid loss of EGDN during the concentration step and later methyl tert-butyl ether [70]. The recovery of organic gunshot residue from swabs can be obtained with different techniques. The main problem is using a minimum amount of solvent, in order to avoid problems associated with concentration of the sample like impurities present in the solvent and loss of the more volatile compounds. An old approach was direct

extraction [69], washing the swab with small portions of ether, resulting in a total volume of extracts of 12 ml. Later, the compression of the swab in a test tube with a narrow hole in the bottom, in a syringe or against the wall of a vial using forceps produced a total volume of about 5 ml. Following swabs centrifugation, over 70% of nitroglycerine (NG), TNT and RDX could be extracted in about 1 ml of the solvent and about 50% with the first spin [71]. It is also possible to use Teflon membrane filter during centrifugation and separate the inorganic GSR particles to be examined by SEM-EDX [72]. Northrop and Mac Crehan [73] tested some sampling procedure to be used for subsequent micellar electrokinetic capillary chromatography (MEKC).

Vacuum lifting is generally used for sampling gunshot residue and explosive particles from clothing, inside of bags, pockets, etc. Jane et al. [74] used a glass-fibre disk in a syringe barrel attached to the laboratory vacuum line. Residues were then extracted with ether. The Forensic Science Laboratory of Northern Ireland developed an efficient vacuuming system for the recovery of organic and inorganic cartridge discharge residue (CDR) [72]. After sampling and extraction, ether can be easily evaporated to near dryness (5–10 μl) using a stream of nitrogen, allowing the last traces of ether to evaporate at room temperature to avoid loss of EGDN [69]. Addition of a small percentage of a non-volatile substance like ethylene glycol (boiling point = 198 °C) prevents losses during the evaporative concentration [73]. The use of a heated centrifuge system to remove ethanol by controlled evaporation under reduced pressure was also proposed [75].

Collection by *tape lifting* allows a preliminary evaluation of the lifted material using a binocular micro-stereoscope, looking for particles to be extracted. The entire tape or a section can be later extracted by ultrasonic agitation [79, 77].

If particles of suspected explosives are spotted, they can be tested on-site by colour tests [78, 79]. Based on the diagnostic field test approach, cost effective devices which can easily be operated by military or law enforcement personnel were developed. A recent publication describes a paper-based analytical device (mPAD) for the detection of components present in pyrotechnic devices and primer residue (Ba, Zn, Al, Fe, Mg, Pb, Ba, Sb, Zn) in real samples. Limits of detection of the metallic components were between 0.025 and 0.4 mg [80]. A simple, sensitive and selective colorimetric paper sensor has been developed by Arshad et al. for detecting nitroaromatic explosive by nanoparticles having an Au core, covered by an Ag shell, functionalized with β-cysteamine. The assay allows rapid on-site detection of TNT in less than 10 min with a detection limit of 0.35 μg/mL. The test has also good selectivity as it does not show interference by other nitroaromatic compounds with similar structures such as dinitrotoluene (DNT), trinitrophenol (TNP), and nitrobenzene (NB) [81]. Bagheri et al. recently developed a mimetic Ag nanoparticle/ZnMOF nanocomposite based colorimetric detection approach for peroxide explosives. H_2O_2, produced by hydrolysis oxidize 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of a new peroxidase enzyme mimetic nanocomposite, based on the Ag nanoparticles embedded into the nano-pores of flake-like Zn-MOFs. The absorbance at 652 nm linearly increases between 0.4 and 15 mg L^{-1} TATP concentration [82].

Explosives having high vapour pressure [83] such as TATP or NG may be analysed after direct headspace, by adsorption on a suitable adsorbent (e.g. Amberlite

XAD-7 and Tenax), by solid-phase microextraction (SPME) [66, 84, 85], by capillary microextraction of volatiles (CMV) [85] or planar solid-phase microextraction (PSPME) [86, 87]. A passive air sampler for the qualitative detection of a wider range of volatile explosives-related components, using thin film sorbents, on chemically and thermally stable substrates, was recently tested on 15 explosive components of mid-high vapour pressures (cut-off at ~10–9 atm at 25 °C) with uptake as early as 1–4 h and retention of analytes on sampler for up to 7 days [88].

When considering vapour phase analysis of explosives, it is important also to recognise the importance of canine units in security activities [89]. Training programs of dogs generally use the real odour but synthetic substitutes are now commercially available for some substances [90, 91].

Moving to clean-up of samples, many procedures were proposed, beginning with the use of thin layer chromatography on silica plate by Twibell et al. [92].

A complete sample extraction and preparation procedure were devised in the nineties both for debris from a bombing scene and for swabs, which were treated with acetone for at least 2 h in a soxhlet extractor; the acetone extracts were concentrated on a water bath at 50 °C and diluted 1:10 with water before clean-up using commercial solid-phase extraction (SPE) columns RP-18. Recoveries were 72% for RDX, 82% for TNT and 94% for PETN [93]. SPE cartridges containing divinylbenzene/vinylpyrrolidone copolymer meet the QA/QC requirements of EPA method 8330 for the 14 compounds of EPA method 8330, allowing recoveries greater than 89% [94]. Recently published sample cleanup procedure for soils and post-blast residues containing nitro-organic explosives such as EGDN, dimethyl dinitrobutane (DMDNB), 4-nitrotoluene (4-NT), NG, 2,4-DNT, 2,4,6-TNT, PETN, RDX, 2,4,6-trinitrophenylmethylnitramine (tetryl), cyclotetramethylene tetranitramine (HMX), erythritol tetranitrate (ETN), and cyclotrimethylene trinitrosoamine (R-salt) are still based on SPE [95, 96]. Extraction procedures of explosives from water were recently reviewed by Veresmortean and Covaci [97].

15.7 Chromatography and Electrophoresis

Thin layer chromatography (TLC) is one of the simplest and most widely used chromatographic techniques and it is extensively used in explosive analysis [64, 60, 66]. TLC is rapid and inexpensive; it can be performed outside a laboratory in open air with simple equipment and permits the analysis of different samples simultaneously time using the same plate. When considering on-site analytical approaches, both colour tests and TLC should always be carefully considered in comparison with more modern but more expensive instruments, often larger, heavier and needing power supply.

Gas chromatography (GC) is a well-established analytical technique in organic chemistry. Capillary columns allow the separation of highly complex mixtures due to their high efficiency and different detectors have been developed to be used with GC, resulting in selective and sensitive methods based on GC, which is often the method

of choice for thermostable substances with a suitable boiling point. The relatively high temperatures employed during the GC analysis could lead to decomposition of thermally labile compounds. For this reason the compatibility of GC with the analysis of explosives is counter-intuitive. Nitroglycerine has a deflagration temperature of 200 °C [5] but the initiation temperatures of single smokeless powder flakes range from 165 and 193 °C and the combustion temperatures range from 184 and 198 °C [98]. Kolla showed that the response of a GC for nitroglycerine decreased raising the injector temperature from 80 °C. The optimum temperature for explosive analysis including RDX and PETN was 170 °C [99]. Moreover, due to the low vapour pressure of many explosive substances, taggants such as 2,3-dimethyl-2,3-dinitrobutane (DMNB), ethylene glycol dinitrate (EGDN), p-mononitrotoluene (MNT) or o-MNT are added to explosive products, allowing easier detection thanks to their high vapour pressure [100, 101]. The possibility to inject large volumes in the GC system allowed significant improvement of the limit of detection of analytical procedures [102]. The most recent approach in GC analysis is the two-dimensional (GC×GC). Stefanuto et al. [101] used a semi-polar BPX-50 column in the first dimension (1D) and a BPX-5 column in the second dimension (2D) for explosive headspace profiling by SPME followed by fast GC×GC-TOFMS.

The GC×GC approach does not provide significant added value when the purpose of the analysis is the identification of a specific chemical substance but has an improved capability in profiling complex mixture. Tsai et al. [103] differentiated production batches of plastic explosives by GC×GC-ToF MS. It is important to emphasise that the level of expertise in the statistical tools needed to carry out interpretation of GC×GC data is too high to allow this approach in routine analysis [104].

High Performance Liquid Chromatography (HPLC) is the method of choice for thermally labile, non volatile or very high-boiling-point compounds. The United States Environmental Protection Agency (EPA) method 8330 for the trace analysis of explosive residues in water, soil, or sediment matrixes was based on HPLC [105]. In the nineties the Forensic Science Service Laboratory of Birmingham screened samples for explosives by HPLC coupled to a pendant mercury drop electrode-(PMDE). The peaks of interest were trapped and confirmed by GC coupled to the thermal energy analyser (TEA) detector [106]. The introduction of MS before and later of tandem mass spectrometry (MS-MS) permitted more selective procedures for forensic identification of explosives. Casetta and Garofolo presented in 1994 a work featuring HPLC-MS-MS parent-ion scan experiments. HMX, EGDN, RDX, NG, TNT, tetryl, DNT, PETN, hexanitrostilbene (HNS), triazido-trinitrobenzene (TNTAB), tetranitroacridone (TENAC), hexanitrodiphenylamine (HEXIL), nitroguanidine (NQ) with an atmospheric-pressure ion source [107]. Today, most of the published methods for the analysis of explosives, based on GC or HPLC, uses MS in combination to enhance reliability of detection.

There is a special attention to ion chromatography (IC), due to the widespread use of pyrotechnical mixtures, containing oxidisers such as nitrates chlorates or perchlorates [108].

Micellar electrokinetic capillary electrophoresis (MEKC) provided very rapid and efficient separations (more than 100.000 theoretical plates/meter) of high boiling point and thermolabile compounds such as explosives in the beginning of the '90s [109, 110]. At the end of that decade, Bailey and Yao showed the applicability of capillary electrochromatography to analyse 14 nitroaromatic and nitramine explosive compounds, using a capillary filled with non-porous particles of stationary phases used in HPLC. A separation with a baseline resolution was achieved with ODS for all of the compounds of EPA method 8330 in under 7 min, featuring efficiencies of over 500.000 theoretical plates/meter. Using more aggressive running conditions, 13 out of the 14 compounds analysed were separated in less than 2 min [111]. Traditional capillary electrophoresis (CE) was used to analyse cations and anions from inorganic salts contained in explosive mixtures [112–116]. Analytical procedures for explosives based on both CE and MEKC were recently reviewed by Calcerrada et al. [117]. Veresmortean and Covaci [97] recently reviewed GC, HPLC and MEKC procedures to analyse explosives in water.

15.8 Ion Mobility Spectrometry and Mass Spectrometry

Ion Mobility Spectrometry (IMS) is a high sensitive analytical technique able to detect a wide range of chemical compounds (both organic and inorganic) at trace levels in gas phase. It was first introduced by Cohen and Karasek in 1970 under the name plasma chromatography [118, 119]. An IMS apparatus is capable of measuring the ionic mobilities of compounds in the gaseous phase and at atmospheric pressure, under the influence of a weak electric field. Most of the applications of the IMS are in the military, security and forensic fields (chemical warfare agents, explosives and illicit drugs) [124]. However, there is an increasing trend towards the civilian applications, like industrial process monitoring and detection of pollutants. IMS features include solid sample introduction on a swab or filter, fast analyses, high sample throughput, high sensitivity, ease of use. The IMS instruments can be rugged enough to be field-portable and easy to use to enable non-scientific personnel to operate it under strictly controlled conditions. For these reasons it is widely used not only in laboratories but on the scene of crime, in airports control and custom zones too. IMS has become one of the key analytical techniques used to detect concealed explosives, including detection both of the vapours and of low-vapour-pressure particles trapped by suitable filters [121]. Particles of explosive with low vapour pressure on surfaces can be collected using wipe samples, made with filter materials, and thermally desorbed into the IMS apparatus. Another way to volatilise explosive materials for analysis by IMS is laser desorption [122]. In conventional apparatus a ^{63}Ni emitter permits the ionisation of analytes mixed with a carrier gas (purified air) and a reactant gas, which enhance ion formation in the ionisation region [123, 124]. The possibility of using negative corona discharge as the ionisation source for negative IMS was studied as well [125]. Laser multiphoton ionisation (MPI) was also used to produce ions from explosive vapours at atmospheric pressure in air for analysis

by IMS too [126]. Another non-radioactive ion source, called variable ionisation potential (VIP) or electron lamp, was developed to replace radioactive ionisation sources [131]. Probably the latest source proposed to avoid regulatory limitations related to a radioactive ionization source is the dopant-assisted thermal ionization, not producing the interfering ions common in corona discharge [128]. During the past decade, improvements have occurred in IMS on the understanding of reactant gas chemistries, the influence of temperature on ion stability and sampling methods [129]. Trace analytes vapour after ionisation are characterized by determining the mobilities of the ions in a weak electric field of an ion filtering zone, called drift tube. The different ions travel in the ion drift tube at ambient pressure and in the presence of a counter current drift gas. The ions with a higher mobility traverse the drift region in a shorter time. The plot of the ion current intensity of the detector versus time, recorded by the control section, gives rise to an ion mobility spectrum or plasmagram. The ion mobility spectrum of an analyte can be characteristic and identifiable. Unfortunately, IMS spectra can contain compounds having similar behavior as the target molecules, interfering with the analysis [130]. Rapid temperature programming coupled with chemometrics was shown as a useful tool for the separation of analytes from interferents [131]. The low detection limits are provided by efficient ionisation process, optimised drift tube conditions and sensitive ion detectors [132]. Filter material properties such as pore size, surface roughness and porosity; flow rate and explosive vapour pressure are parameters that can affect the IMS response [133]. A special attention was soon paid to application of IMS in airport security activity. The coupling of an airline passenger personnel portal with a high-flow (HF), high-resolution (HR) IMS was described [134]. The first study to identify possible interfering air contaminants common in airport settings by IMS was published in 2001 [135].

Solid phase micro-extraction (SPME) methods were combined with ion mobility spectrometry to provide rapid and sensitive qualitative and quantitative analysis of explosives [128, 136, 137]. A manually operated system that can detect 2,4,6-trinitrotoluene (TNT) in seawater at a concentration of 0.010 parts-per-trillion in less than five minutes was developed by Rodacy et al. (2002) [138]. IMS was also coupled to an electrospray ionisation source to detect explosives [139].

IMS is particularly suitable to security applications, when a fast and sensitive detection and a low FAR are necessary. However it lacks the selectivity needed to provide evidence in court. MS based analytical methods provide better sensitivity and selectivity but are generally more time consuming, often requiring an upstream separation such as GC or HPLC. The use of a mass spectrometer as a detector allows for adjustable selectivity, based on the number of ions detected. Monitoring only one ion (m/z 43) it is possible to develop a sensitive detection method for TATP, allowing a limit of detection (LOD) of 10 pg/mL [140]. The LODs for TATP by GC/MS measured considering the three ions at m/z 43, 59, 75 (in order of abundance) reported by Sigman et al. were 100 pg by ion trap MS and 50 pg by quadrupole MS [141]. Samples giving a positive result by monitoring a single ion, can be later analysed following three ions by GC/MS to confirm the presence of TATP providing forensic evidence. TATP can be also analysed HPLC/MS using an atmospheric pressure

chemical ionization (APCI) interface, operating in positive ion mode, allowing limits of detection in the low nanogram range [142, 143].

Nowadays a special attention is paid to ambient MS [144] such as desorption electrospray ionisation (DESI) [145], direct analysis real time (DART) [146, 147], dielectric barrier discharge (DBDI) [148], low-temperature plasma (LTP) [149], and paper spray-mass spectrometry (PS-MS) [150]. Sensitivity levels are at nanogram to sub-nanogram level but they are strongly dependent on the presence of confounding species and matrices. This means that there is the risk of false negative results. Moreover, quantification can be problematic due to different issues, including matrix effect, especially with complex mixtures. Also the reproducibility and inter-laboratory repeatability of ambient MS analysis can suffer inconsistencies. The two major issues are the sampling approach (i.e. vapour analysis, swabs) and the lack of an inclusive database for ambient MS explosives spectra [151, 152].

15.9 Conclusion

The 2017 Annual List of Explosive Materials by the United States Bureau of Alcohol, Tobacco, Firearms and Explosives included more than 200 chemical substances and mixtures. This means that the general issue of the explosive threat is too complex to expect a single analytical tool to be comprehensive enough. There is not and it is not expected a "magic wand" both in security and in forensic applications. The strategies need to be developed with a fitness to purpose approach, based on several aspects such as probability of a false positive, probability of a false negative, analysis time and price. Moreover, in security applications, the option of remote sensing (stand-off) is particularly desirable.

If new tools made available by advanced analytical chemistry are to be applied in security or in forensic science, it is important to consider that they need to provide chemical information with the aim of helping decisions to be taken respecting a juridical framework.

In security, the probability of a false positive can be higher, compared to results providing forensic evidence, because of the limited time available to take decisions. A false positive for a passenger boarding on a plane results in a short delay for a person, who will be examined a little longer. A false negative could be an improvised explosive device on a plane taking off.

When analytical results are used as evidence in court, a false positive would result in an innocent being convicted, which is not acceptable.

The new laser sources are monochromatic, tunable, powerful, capable of short pulses (nanosecond), highly coherent, thus offering the opportunity to realise electro-optical tools capable of stand-off analysis. The wide range of emission (from far UV to IR) offers the possibility to implement different spectroscopic techniques. The high power and the coherence of the laser beam allow analysing targets far away from the laser source and the detector.

Highly powerful laser operated from UV to IR can produce large plasma emission and therefore to retrieve information from atomic emissions, as in case of LIBS technique, to molecular vibrations as in the Raman or photoacoustic spectroscopy.

During last decades, many efforts have been put into implementing such technologies in field applications, with success stories; nonetheless some concerns remain in the calibration procedure that cannot be compared with laboratory tools with well-established protocols. This issue is mainly due to the method of the sample interrogation, typically located remotely from the laser transmitter and not isolated from the background.

Therefore, such optical tools demand for specific spectral databases in order to both accomplish an internal calibration and also to extrapolate the features to be revealed in the remote target. A spectroscopic database is implemented for calibration purpose and to assign the spectral emission to the element or molecular group. This spectroscopy database cannot be univocal and similar to other spectroscopic tools. Furthermore, calibrated targets are needed to avoid external environmental factors affecting the shape and intensity of the spectral lines.

Processing of spectral data requires suitable and ad hoc algorithms to be able to extract the features of interest. The interpretation is typically performed with Principal Component Analysis and the evaluation is referred to a database of spectra obtained with reference samples already characterized in laboratory.

As summarized in Table 15.3, most of the optical techniques offer the advantage to have a direct interaction with the laser radiation, without sampling and prior preparation, simple to use, high Technology Readiness Level (TRL), selectivity and sensitivity with a very cheap cost per single analysis. On the other hand, eye-safe regulations play a severe limitation in external applications.

Nonetheless, technology is continuing to improve the development of new more compact and performing devices that can be profitable implemented in future sensors.

The analytical methods providing evidence in court are always multistep procedures. They start with sampling, sometimes with the aid of on-site techniques. Items and samples are then transported in the laboratory to be treated and solutions are generally analysed with hyphenated techniques base on chromatography followed by mass spectrometry.

Miniaturisation is giving the opportunity to bring more and more instruments on-site (e.g. instruments for GC, EC or MS). New sources for MS are proposed and

Table 15.3 State of the art in the laser techniques performances

Technique	On-field	Sample preparation	Eye-safe	TRL	Sensitivity	Selectivity	Interferents
LIBS	Yes	No	No	6	ng	High	Yes
LPAS	Yes	No	Yes	6	Trace	High	Yes
RAMAN	Yes	No	Yes	6	100 $\mu g\ cm^2$	High	Yes
SERS	No	Yes	No	3	pg	High	Yes
ILS	Yes	No	No	5	Fingerprint	High	Yes

HPLC is gaining more and more space but the large number of theoretical plates and the high reproducibility when using GC/MS are not yet possible with HPLC/MS.

Improved databases are not only important for identification of chemical substances but also of commercial products, which are often mixtures.

Another interesting subject for scientific research in the field is the need of more effective statistical tools to fully exploit analytical results. This aspect is becoming more and more important in forensic science and implementation in courts will possibly increase if the judges becomes proficient in handling probabilistic evaluation of scientific evidence.

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Chapter 16
End User Commentary on Advances in the Analysis of Explosives

Wolfgang Greibl

Clearly the terrorist's attacks in France in 2015 and Belgium in 2016 is still in everybody's minds. In these attacks over a hundred people were killed and many hundreds have been injured. In addition to the loss of many lives, this has also been considered the worst case possible involving explosives, from the forensic point of view.

Imagine the tireless work of the IED experts searching the scenes of an explosion with a pressing demand for fast and accurate results hampered by the delivery of significant trace evidence to the crime lab. If CSI were able to conduct an informed search, this would help reducing the amount of samples to be analyzed later in laboratory thus focusing on the most promising samples to provide relevant intelligence on time. In addition to bulk search the crime scene, surfaces such as glass, paint, plastics, metals, wires, etc. will need to be collected/analyzed to provide the investigators with clues on what to look for.

From an end user forensic perspective, there are two main areas of interest: the detection of explosives at the scenes of crime and the provision of scientific evidence demonstrating use of explosives, identity of the explosive and circumstances/dynamics of the explosion.

So this is where we start: in the extremely messy crime scene after an explosion. IED experts are the first to examine the area for possible other threats. They have to clear the scene of crime in order to declare it safe for other police officers to start their work.

Imagine the stress to walk into the devastated building, crumbled streets and airports in eighty kilogram heavy protective gear, not knowing where you going to next step or put your hands on. Everything out of the ordinary could be a potential threat. Nowadays counter-IED officers are thankfully equipped with analytical tools to detect and identify explosives.

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Raman spectroscopy (bench top and hand-held devices) are currently the golden standard in the field of explosives. However, one drawback, even with hand held devices, is the necessity to still get in the close proximities with the potential threat.

It would be very advantageous and safer, especially for IED experts to get intelligence on the presence of an explosive device from several meters away.

An interesting technology for the distance detection of explosives is Laser induced breakdown spectroscopy (LIBS).

As technology improves, more powerful measurements can be made from approximately hundred meters, even as attachments to drones in order to scan and detect from various kilometres away (from safe locations).

However, with LIBS and Raman spectroscopy, there is still the need to focus the laser on a very small area, from some square micrometres to square millimetres; with the typically very messy crime a scenes after an explosion, hundreds of square meters to search and several tons of debris lying around, this exercise becomes extremely challenging and time-consuming. In fact, time is a very significant issue as the area needs to be declared safe (or not) as quickly as possible whilst not missing any information to this effect. Another issue with the use of high power lasers is safety, given the high intensity of the used radiation. This is however not just an issue within the international eye safety regulation; the high intensity of the laser shone might also set off sensitive explosives.

Amongst alternative technologies, Infrared photography may be of interest. This technique is widely used in document examination and also in the development of gunshot residues patterns. It would be very interesting to investigate if infrared photography could be used as a screening technique for the detection of explosives; a crime scene could be photographed with a panoramic camera and observed for typical patterns of explosives which can then be confirmed with other analytical methods.

Infrared photography has been further developed by using a number of different wavelengths and one interesting development is multispectral imaging. Once again, the idea is to gather as much information on the scene of crime as possible without being there physically and maybe even getting accurate measurements in order to minimize further explosives threats.

Once the IED experts declare the area safe, the crime scene officers can begin collecting traces at which point it is possible to make more sophisticated and confirmatory measurements.

Of interest to the end users working in the field of explosives detection is the improvement of the Raman signal by surface enhancement with gold or silver nanoparticles (SERS). These devices are also portable and extended applicability to other fields of forensics has been observed.

In terms of remote analyses, there is always the need for more sensitive and reliable methods with preferentially no sample preparation. Direct analysis in real time (DART) coupled to mass spectrometry has shown great promise for explosives detection due to fast and very sensitive analysis. Also, here no sample preparation is needed. It is also of great value that both organic and inorganic explosives can be detected by such instruments thus avoiding the use of multiple platforms for multiples

classes of explosives. In order to correctly identify the explosives, two orthogonal methods should be used to avoid miss-interpretation of the results. A false positive cannot be accepted in court because this might lead to a conviction of an innocent. There is a limited amount of possible analytes so the methods can easily be validated and standards are widely available.

In forensic practice, there is a great need for standardized operations, the validity of which should be maintained across laboratories and countries. Whilst the analytical academic community is working on this (using liquid/gas chromatography couple to mass spectrometry), presently, there is no standardized/validated method covering analysis of explosives. Such protocols could be used in each laboratory to continuously build databases of relevant explosives analytes.

Of more and more pressing importance, are methods enabling the determination of the level of background contamination of explosives. This is especially crucial giving the ever increasing analytical sensitivity of modern technologies.

Activity level information would be extremely important for the correct interpretation of traces of explosives detected on clothes, belonging or premises of the suspects and thus avoid potential miscarriage of justice.

In conclusion, the remote measurement of traces of explosives at scenes of crime is still limited at present. There are many instrument and methods to detect explosives reliably but, as it is in the nature of these incidents, time is limited and every further development that speeds up the gathering of intelligence on explosives is welcomed.

Chapter 17
The Application of Forensic Soil Science in Case Work and Legal Considerations

Lorna Dawson, David Parratt and Derek Auchie

Abstract Forensic soil science is now an accepted discipline in many nations worldwide such as the UK, the Netherlands, Germany, Australia, Russia, Italy, Japan and the USA. Other nations are adopting the approach suitable for their own types of soils, crimes and land cover. There are a range of methods which can be used, with new methods being researched, developed and tested all the time. This chapter will not discuss the specific analytical methods used but instead outlines suggested strategies for examination and analysis and the presentation and communication of these results in court. The methods adopted for analysis in case work will often depend on the case in question, the examiner, the soil type and the individual country and legal system involved. Forensic soil science application can in general be divided into two main areas: (1) for intelligence gathering, such as providing information on search and narrowing down areas of interest, or in crime reconstruction and (2) for trace evidence comparison, evidence provision, evaluation of data and presentation in court. As the area of forensic soil science is relatively well established, this chapter concentrates on legal aspects of the use, acceptance and application of new methods, particularly acceptance and admissibility in court.

17.1 Introduction

Forensic soil science has developed over a long period of time, beginning in Roman times when people used the soil information on the hooves of their enemies' horses to tell from where the enemies had travelled. Techniques and approaches have advanced considerably since that time, although the same general principles remain. This chapter focuses on the current situation in the UK, although forensic soil science is used to good effect in many countries worldwide, including Russia, Japan, USA, Australia, Spain, Germany, the Netherlands and Italy. In some other nations (such as

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in Brazil, Argentina, Belize, China and India, for example) this science is currently under development. Forensic soil science application in the United Kingdom can be divided into two main areas: (1) for intelligence gathering, such as informing on search, or in crime reconstruction and (2) for trace evidence comparison, evidence provision, evaluation and presentation in court. As the area of forensic soil science is relatively well established, this paper concentrates on legal aspects of the use and application of new methods, particularly in court.

Any forensic soil scientist engaged by Law Enforcement or forensic agencies to carry out work should be aware that they may be required to report on their findings and ultimately could be required to present their findings in court. Forensic means relating to or denoting the application of scientific methods and techniques to the investigation of crime/relating to Courts of law [1]. The position with regard to expert witness reporting, court evidence (testimony) and some of the main legal evidential questions, which are likely to affect soil and wider geoforensic evidence, are discussed in this chapter. This chapter will focus on the legal system operating in England and Wales, albeit many of the general principles are applicable worldwide.

Each country has its own unique legal system, developed over centuries, reflective of the political, cultural and historical development of that individual country. Even between countries with very similar legal systems, the precise rules around the leading of evidence will be (although often marginally) different. Forensic soil science evidence can feature in a criminal case in any jurisdiction. It is critical therefore that any method or new approach is developed, interpreted and presented in such a way that it can withstand judicial skepticism and challenges from lawyers, even in the toughest of legal environments.

One of the most challenging environments for any expert evidence is the common law court with its adversarial procedures, such as is in the UK, comprising three separate jurisdictions: England and Wales; Scotland and Northern Ireland. Less challenging are the courts in the inquisitorial regimes. If the evidence can survive in the adversarial system, it is unlikely to encounter difficulty in the inquisitorial arena. Criminal cases of a serious nature are held in front of a jury in the UK, and so experts must be able to communicate their science in a clear manner to legal experts and to the general public (juries). In the UK, expert witnesses may also be appointed by defence counsel to challenge the expert opinions provided on behalf of the prosecution.

Expert work carried out for the court is commissioned by the prosecution or defence or in some cases by the Criminal Cases Review Commission (CCRC). The role of the expert (whichever scientific discipline they are from) is to assist the court in understanding the evidence that they present. The prosecution authorities in the UK (all working in line with the common law tradition) are: England and Wales—the Crown Prosecution Service (CPS), Northern Ireland—the Public Prosecution Service (PPS) and Scotland—the Crown Office and the Procurator Fiscal Services (COPFS).

Standards—When providing evidence, it is important that any authoritative guidance, protocols and approved standards are adhered to. Many forensic methods are accredited by the United Kingdom Accreditation Service (UKAS) to the international laboratory ISO17025 quality standard. This accreditation ensures that forensic labo-

ratories follow standard procedures and encourages the delivery of precise, accurate data and reporting. All other methods followed in forensic geoscience laboratories follow similar quality control procedures and are, in general, covered by the standard, ISO9001. Laboratories carrying out case investigations are encouraged by the Forensic Science Regulator (FSR) to have UKAS accreditation, to help ensure that a minimum acceptable and externally regulated standard is met.

Currently, there is no approved standard for conducting ground searches, although some papers on general guidance exist [2]. The Forensic Science Regulator (FRS) UK works with the UK Home Office and ensures that the provision of forensic science services across the criminal justice system is subjected to an appropriate regime of scientific quality standards. The FSR also collaborates with the authorities in Scotland and Northern Ireland who have expressed their willingness to be partners in the setting of quality standards which will be adopted within their justice systems. This, at time of writing, is a very important topic across the forensic science disciplines, with all areas having different issues. The House of Lords has undertaken an inquiry on the topic of forensic science provision in England and Wales (https://www.parliament.uk/forensic-science-lords-inquiry).

Ethical context—Adherence to high moral and ethical standards is also of paramount importance for the practising of forensic soil science/geology: fundamental to settling issues related to crimes against people, society, our built environment, our natural environment and our heritage. The geosciences face legal aspects related to offences and crimes of various and many types, of a civil and criminal nature.

There is a need for practitioners in this area to ensure compliance with appropriate ethical standards in their work, in order to protect themselves, their workplace, their profession, the community, customers, and the built and natural environment. Forensic soil science also relates to aspects of engineering geology, of particular importance in civil cases. Geoethics is an emerging subject within geoscience research and practice which promotes an ethical way of thinking and practising the geosciences, within the wider context of the social role of geoscientists. It aims to improve both a high quality of professional work and emphasizes the important acceptable social credibility of geoscientists, to foster excellence in the geosciences, and to assure sustainable benefits for communities under a scientific perspective. It should protect local and global environments and create and maintain the conditions for the healthy development of future generations.

Soil—Soil science is a fundamental part of the geosciences. Soils are composed of both inorganic (i.e. minerals, elements) (Fig. 17.1) and organic (i.e. primarily plant derived) constituents (Fig. 17.2). The approach taken and methods chosen when characterizing soil in forensic case work often depend upon the availability of the equipment, costs, resolution, as well as the size and condition of the questioned samples available. Consideration also should be given to the destructive nature of an analysis technique and the sequence of analysis. It is essential to carry out non-destructive analyses before the sample is destroyed. In addition, consideration should be given to the potential strength of evidence which depends upon the type of analysis, the number of measurements made [3] and the complementarity of such measurements [4].

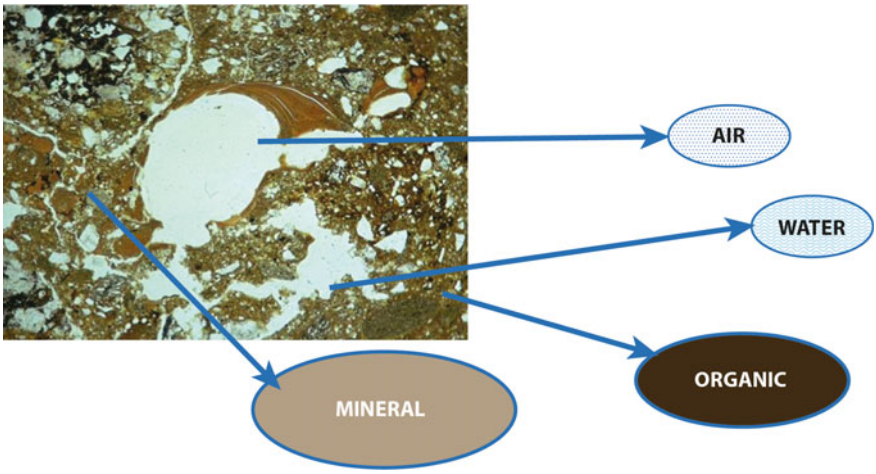

Fig. 17.1 Soil composition: inorganic and organic components in an annotated impregnated thin section of soil

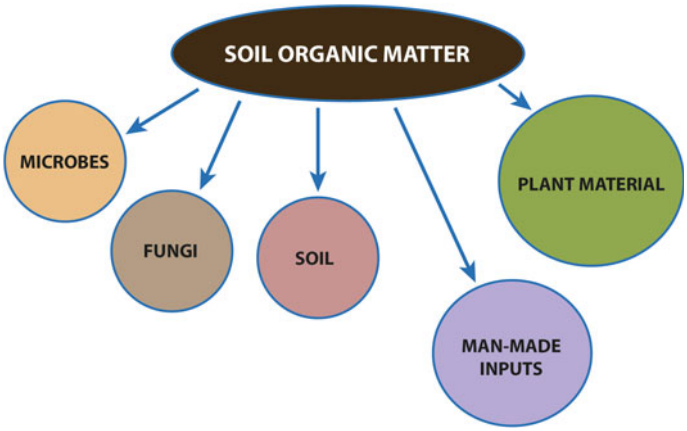

Fig. 17.2 The range of some of the main soil organic matter types used in forensic characterization

Historically, inorganic methods such as chemical and mineralogical approaches have been most generally applied in casework. Persistent biological approaches, such as the use of palynology or wax marker analysis, has also been used in some countries (e.g. UK, New Zealand, and The Netherlands), either in addition to the characterization of the inorganic component, or on its own, often when a restricted sample is available. The combined approach provides a greater evidential value. It is recommended that the two distinct phases of soil (organic and inorganic) are both characterized where sample size permits to provide the best possible comparability of any two samples [4]. Recently, however, analysis of individual largely single

source aggregates has allowed greater comparator analysis involving soil organic matter (such as presented in evidence in UK court cases *R v. Muir*, 2013, *HMA v. Sinclair*, 2014 and *R v. Halliwell* and *R v. McKie*) [pers comm, Dawson]. The recovery and analysis of the individual aggregate approach (Fig. 17.3) minimizes the risk of contamination and reduces the issue of the questioned sample possibly originating from multiple sources, i.e. a mixture, which makes any comparison with a scene sample (known location sample, sometimes referred to as control or reference samples) difficult.

Questioned soil samples (those from an unknown location, such as a suspect's shoe, vehicle, spade etc.) can often be very limited in size, which may restrict the range of options for analytical methods to be used. In addition, these samples may contain many more materials than are considered 'natural' components of soil, e.g. brick, fly ash, cement. The anthropogenic source material can, however, be useful in comparing two soil samples, particularly when the material is unusual in nature. Transfer and persistence of material has also to be considered when evaluating any results, and careful assessments must be made of the data so that similar size fractions are compared in any analysis [5]. The moisture content of the soil at time of transfer is also important for transfer, as is the condition of the contact location soil and the depth of contact with, for example, a puddle on the road.

Detritus picked up from urban pavements and street gutters, as well as introduced material such as faecal material of various origins (e.g. animal, human, bird) can also be analysed using the same combined inorganic and organic component approach [4]. The broad spatial variation in soil, roadway, water, building materials, and air and water borne particles can be contrasted with the variation in urban materials, from dwellings to streets or parks or gardens, along with micro-spatial variation in each [6, 7]. Microplastics are another group of materials that can potentially be included in

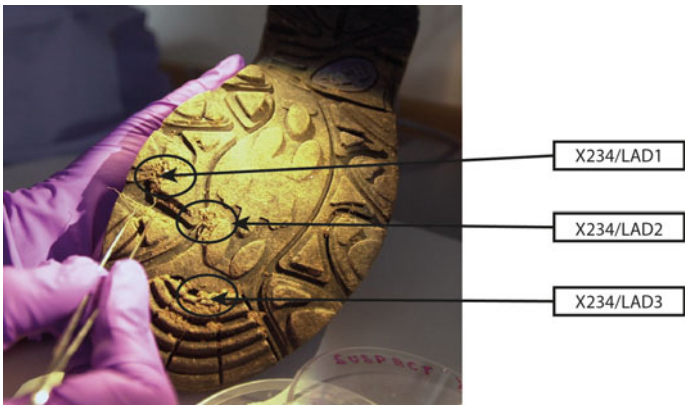

Fig. 17.3 Image of the sole of a suspect's shoe (X234) showing visually distinct and likely different source material adhering (LAD1, LAD2 and LAD3). These three different samples were recovered and analysed separately thus increasing the chance of single source comparison with a known source or sources

the multi-component characterization approach which can help increase evidential strength of any comparisons made of the trace evidence. One issue with including such anthropogenic particles is the current lack of relevant databases which makes any estimate of likelihood very difficult.

17.2 Case Work, Search

Most intelligence work involving forensic soil science is carried out directly for police forces. This is predominantly in the role of assisting with ground search operations for missing people, unmarked homicide graves and for concealed objects. This uses predictive geolocation in narrowing down areas of potential search. Predictive geolocation is the use of the physical, chemical and biological attributes of a soil sample in the identification of its provenance (e.g. sand grain shape, metal concentrations, vegetation fragments, etc.). The overall aim of predictive geolocation is to determine the area from which a soil sample was most likely derived [8].

Many aspects of the texture and composition of a soil sample have spatial significance, and the more of these which can be determined, the clearer and more spatially resolved the geographical profile will be [e.g. 9]. The precision of such spatial predictive analysis often depends upon the quality and spatial resolution of the associated databases. In the UK, there are very good soil (James Hutton Institute, Cranfield University) and geological (British Geological Survey) maps as well as digital databases. Often, the attributes measured on the soils in the available databases will have been for agricultural, environmental or exploration purposes; they will not have been collected at the most suitable depth and are thus not ideally suited for forensic application. Such geolocation analysis will therefore often not point to a specific single location (X on a map) and investigators need to be aware from the outset of any location work that the analysis is in most cases unlikely to allow a discrete specific location to be determined. Instead, an environmental profile can be established which is likely to include: (a) the soil types present in the area, e.g. sand, loam (b) the nature of the underlying bedrock geology and any superficial deposits, e.g. granite, sandstone (c) the nature of chemical and physical processes operating in the area, e.g. erosion (d) vegetation cover and a description of the overall habitat(s), e.g. heathland, coniferous forest (e) the geographical ranges of biological species identified in the soil sample e.g. upland, coastal (f) abundance and types of man-made particulate materials (plastics, fly ash particles) and their location significance along with other data in determining the likely human activity and processes in the source area.

Many of these parameters can be overlain using spatial data (often in a Geographic Information System model) to narrow down and focus the description of the likely source area. Geographical Information Science (GIS) is routinely used in the UK to integrate and analyze the different types of geocoded data with information from databases useful to forensic investigations. An example of this is where a test questioned sample was submitted for analysis with no other information avail-

able, other than it was somewhere in Scotland, and the location of where it had originated was pinpointed to within 800 m of where the test presenter from BBC4 had walked[1] (Fig. 17.4). Working with the law enforcement agencies, the impact of such approaches has resulted in a more informed and integrated approach which has enabled a higher degree of quality assurance and a prioritization of potential search areas. Forensic geoscience is a niche discipline, not provided within the larger forensic service providers, but usually subcontracted to individual experts with relevant experience and qualifications. This environmental profile can then be used by the investigating officers in conjunction with other layers of evidence (e.g. automatic number plate recognition (ANPR), mobile phone use analysis, eye witness accounts, line of sight analysis, etc.) to help focus search assets in potential target areas. Whilst the derived geographical profile is by nature generic, it also means that many other locations with different environmental profiles can be excluded as the likely source for the questioned soil (i.e. the SIO can be confident that the questioned soil could *not* have come from any areas of clay soil, or woodland, or arable or nor is it close to habitation, etc.). Once this data is mapped and spatially constrained using the available police intelligence, often only one or two areas are consistent with all the measured required attributes and thus can help prioritize areas for search activity saving valuable time and resources.

Such an approach has been adopted in the USA [10]. One example of this "geo-provenancing" approach used in the UK in search was in the search for murder victim Pamela Jackson on the moors near the M62 [11]. The victim's body was located using comparative soil analysis of material brushed from the back of a pair of gloves found in the boot of the suspect's car with possible sites where the suspect's car had potentially been parked. Another example is the successful location of a grave in the Pennines, England, where the victim's body had previously lain undiscovered for at least 10 years [12]. UK geoscientists have also provided formal and informal advice, training and operational support for law enforcement led searches throughout Europe, Asia, Africa, Australia, Canada, USA and Latin America.

17.3 Case Work, Trace Evidence

In the UK, forensic soil science is used mainly in cases of serious crime such as murder, rape and in ground searches associated with organized crime or counter terrorism. Different techniques and approaches have been used, depending on the individual case in question and on available laboratories. For example, microfossil and pollen analysis was used in the *R. v. Ian Huntley* double murder case where the evidence was used to show that Ian Huntley had driven his car on the remote track where the bodies of his child victims Holly Wells and Jessica Chapman were found in 2002. A complementary approach was adopted using both inorganic and organic soil analysis as trace evidence provision in the search, recovery and in the trial of Adrian

[1] https://www.bbc.co.uk/programmes/p02l4px7.

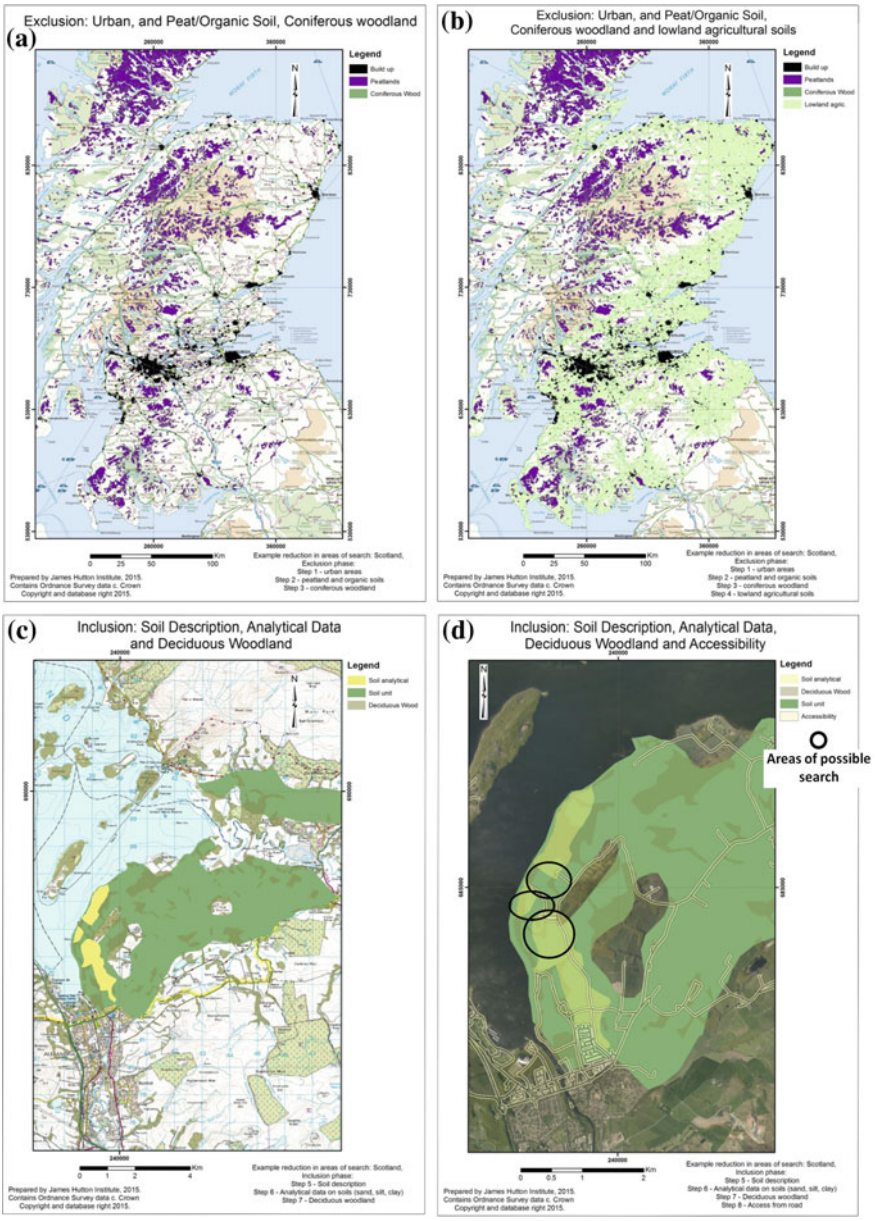

Fig. 17.4 Example Geographical Information Science (GIS) maps produced from a single questioned soil, examined under light microscopy, for organics, mineralogy and texture to narrow down areas of search in Scotland. **a** Exclusion of soils which are urban, peats or coniferous woodland soil, **b** exclusion of areas of lowland agricultural soils, **c** inclusion of areas of soil texture, pH and deciduous woodland and **d** inclusion of accessibility factors such as distance from road. *Maps produced by David Miller, James Hutton Institute*

Muir (*R. v. Adrian Muir*, 2013). In the murder of Rebecca Godden, culminating in the trial of Christopher Halliwell in 2016 (*R. v. Christopher Halliwell*), soil recovered from tools in Halliwell's garden shed was virtually indistinguishable from soil found at the grave site where Rebecca Godden was discovered (pers comm, Dawson). In Scotland, in the investigation of the Worlds End murders and associated trial in 2014 (*HMA v. Angus Sinclair*) soil samples from the feet of one of the victims (Helen Scott) were shown to contain wheat grains and characteristics of the grass verge and the wheat field where Helen Scott was found murdered in 1977 (pers comm, Dawson).

Forensic soil science is generally a comparison between two or more samples to ascertain whether they likely originated from the same, or alternatively, from different sources. When any two soil samples are indistinguishable in measured characteristics, the possibility that they originated from a single source cannot be eliminated. Such evaluation of comparability between samples requires expert opinion. The samples can be excluded, however, as coming from a common location if their analytical characteristics are different. The use of a comparison with database values is very important for this, in particular relevant context databases, and the possibility of applying Bayesian statistics or likelihood ratios being calculated (see evaluation below) [13].

Whilst we do not advocate the use of a standard soil analysis or a standard operating procedure (SOP) across the world, there are several general guidelines which would usually be expected to be followed in a lab carrying out forensic soil science examination and analysis. In general, most examination and analysis will start with the broad scale and subsequently become more focused at a more detailed scale—for example a visual examination of the soil trace would be carried out first, followed by examination using binocular microscopy and then a more detailed chemical, biological or physical analysis. Initial visual or binocular microscopy is commonly required before the subsequent analytical pathway is recommended to the investigator. Samples submitted to the forensic soil scientist for analysis can be variable with a consequence being that the analytical pathway required will differ enormously between cases. Soil samples are usually composed of a mix of (i) naturally occurring inorganic minerals and elements and particles, (ii) organic materials, including organic particles such as plant debris, spores, pollen, vertebrates and invertebrates and micro-organisms (including micro-fossils) and (iii) man-made particulates such as metals, plastics, hairs, fibres, paint, etc. Depending upon the relative amounts of these three phases present in a soil being examined, different analytical approaches will be recommended to the investigating authority and subsequently applied. As a result, the initial examination of soil samples is a very important stage (not least to ascertain how much material is present in the questioned sample) which will exclude the use of some methods which require a certain minimum size of available sample e.g. Inductively Coupled Plasma (ICP), or quantitative X-ray diffractometry (XRD). Sample mass will usually constrain what analyses can be carried out and as the questioned samples can be significantly less than 1 g, methods which can be applied to trace amounts of samples and provide quantitative values are advantageous. The

methods chosen to use in each individual case will be the ones which best address the questions being asked of the sample on each individual case question.

The general procedure is one of starting with non-destructive methods followed by destructive methods. In addition, it is good practice to retain a portion of sample for possible subsequent re-analysis or retesting by defence experts. At the initial stages, visual methods are therefore an advantage, as are spectral methods such as Infra-red spectroscopy or micro-spectrophotometry. Initial screening will not provide quantitative data but will significantly influence the analytical pathway. This is an essential stage in the description of a sample and should ideally be documented through the collection of representative digital images. Evidentially, visual screening of a soil sample may be the only analysis required as it may demonstrate that questioned samples are clearly distinct (different) to reference samples from the scene (although the true variability at the scene needs to be known to safely draw this conclusion). A multi-proxy approach for analysis is better than relying on a single method and one based on inclusion, and where possible, to include both phases of inorganic and organic [15]. If multiple, independent or semi-independent variables within a single sample can be measured, then this will strengthen the degree of confidence in any subsequent evaluations made and conclusions drawn of comparability. In some cases, a multidisciplinary approach will be required, which can be used to independently test any conclusions drawn (botany, geology, palynology, etc.). Some methods can be used to effectively exclude a comparison between two samples, but they are not sufficient analytical to determine that a questioned and known sample were derived from the same place. For example, if a questioned soil is grey in colour and sandy in texture whilst the known reference soils from the crime scene are all orange in colour and are clay in texture (and they all have the same moisture content), then it can be concluded that the grey sandy soil has not been derived from the crime scene (orange clay) and can be excluded as sharing a common origin. However, even if two soils are both grey in colour and sandy in texture, this information is insufficient to conclude that they necessarily came from the same location. In addition, it is important to make a comparison of similar fractions of the questioned and known soils. For example, if a clay fraction has been transferred to an item of clothing, then the clay fraction from the reference samples at the crime scene should also be separated and subsequently analysed, not the whole soil. This is important as on many occasions the questioned soil can be a mixture of sources (e.g. deposited over time on a wheel arch or recovered from a foot well mat), and thus we advocate recovery of individual aggregates with similar colour and texture from any questioned item under examination. The range of analytical methods available for the forensic soil scientist to potentially use is huge [14] and is increasing as new methods are validated through research. However, prior to being used in evidence any new methods must have been thoroughly tested, peer reviewed and the new approach accepted by peers.

17.4 Evaluation of Soil Data

Despite being widely discussed across many forensic disciplines, probabilistic reasoning within a Bayesian framework has had very limited penetration within the discipline of forensic soil science or geoscience. Current advances within the discipline have a strong focus on the application of increasingly sophisticated analytical methods to discriminate between different soil or geological trace sources. While analytical advances may well be important additions to the tools available to the forensic geoscientist, the development of a probabilistic approach to underpin an expert's opinion based on the outcomes of these analytical techniques may be useful in addressing some of the key issues within the growing debate over approaches to such evidence. In the past, various methods have been used for evidence evaluation: numerical and verbal scales [3] exclusion principles (as used by the US Department of Justice) [15] and indices of comparability [16]. In evaluative mode, in a Bayesian framework approach, an appraisal of a likelihood ratio (LR) for the scientific observations is offered as a measure of the weight of the evidence.

The role of the expert in this evaluative mode is to help the decision maker(s) in a court of law arrive at a decision on the questions of provenance (source) or activity—it is the expert's role to assist the court in coming to the best decision. Application of this interpretative framework in forensic soil science, as becoming adopted in other forensic disciplines, could prove useful in advancing forensic soil science by helping to define relevant populations from which to collect data, guiding the design of forensic research studies, facilitating the combination of the results from multiple analytical techniques and providing a common approach to interpretation and the communication of expert soil science opinion. However, before this can be achieved, the relevant and representative databases will be required to be made available.

17.5 Report Writing and Presenting Evidence

Although a report should adhere to general guidance on expert reports (see below), the information and structure of a witness report is often in an expert's own style. However, there is certain mandatory content for expert reports, and these will vary depending upon the jurisdiction (country) in which the crime has been committed. These requirements (where they exist) should be closely followed by the expert. Where in doubt, advice on any such requirements should be sought from the police or lawyers in the relevant country.

The other important consideration is adherence to good practice. There is some advice, albeit in the context of civil cases, from the UK Civil Justice Council. Although references to the Civil Procedure Rules (CPR) will not apply in criminal cases, some of the general advice on the instruction of experts and the content of expert reports is relevant in both civil and criminal cases.

In this chapter, the English and Welsh jurisdiction is focused on. The Civil, Criminal and Family Procedure Rules, Practice Directions and the Guidance in English law [17] do all have requirements which must be followed. In particular, certain declarations and statements must be included in the reports. There are rules for Criminal Proceedings too in relation to experts. Rule 19.2 of Criminal Procedure Rules (CrimPR) provides for "Expert's Duty to the Court": https://www.justice.gov.uk/courts/procedure-rules/criminal/docs/2015/crim-proc-rules-2015-part-19.pdf.

Rule 19.2 describes the expert's duty to the court, rule 19.3 the introduction of the expert evidence, rule 19.4 the report content, rule 19.6 prehearing discussion of expert evidence, rule 19.7 single joint expert rules and rule 19.8 instructions to a single joint expert, with rule 19.9 describing the Court's power to vary requirements under the Part. Although these are only applicable to England and Wales (there is no Scottish equivalent, and rules in other jurisdictions will be different) they are a useful guide, especially on content (rule 19.4 in particular). Many of the principles will apply to other jurisdictions and can be used, for example, if there is no precedent available from the requesting authority.

The Criminal Procedure Rules are continually reviewed. For example, amendments came into force **on April 1st 2019** whereby under amendments to Criminal Procedure Rules 19.2 and 19.3, experts have a duty to disclose to those instructing them anything of which they are aware which might reasonably be thought capable of:

- undermining the reliability of the expert's opinion, or
- detracting from the credibility or impartiality of the expert.

The party instructing them will have to serve notice on the other side, together with the expert's report, of anything which falls under the categories above. The original 19.3(c) required disclosure, by the instructing party, of anything reasonably thought capable of "*detracting substantially from the credibility of that expert*". There was, however, no explicit duty on the expert to disclose to their instructors. The new wording focuses on matters affecting the reliability of the expert's opinion and the credibility and impartiality of the expert and places a clear duty on the expert to make that disclosure.

Accreditation. When providing evidence, it is important that authoritative guidance, protocols and approved standards are adhered to (see section above). Practising forensic geoscientists in the UK may become Chartered Scientists or register through their own professional societies or through membership of their professional organizations (e.g., the British Society of Soil Science, the Geological Society). In addition, individual scientists can register with the National Crime Agency (NCA) if they are invited to do so by the police.

The FSR UK works with the UK Home Office and ensures that the provision of forensic science services across the criminal justice system is subject to an appropriate regime of scientific quality standards. The FSR also collaborates with the authorities in Scotland and Northern Ireland who have expressed their willingness to be partners in the setting of quality standards which will be adopted within their justice systems. The FSR is also represented on some of the working groups.

17.6 Court

The jury in an adversarial system consists of lay-persons. The duty of the expert is to assist the court in the area of their expertise and the court will consider that evidence in deciding the case and whether the case has been proved to the requisite standard and burden of proof. The expert's role is to provide independent assistance to the court by way of objective, unbiased opinion in relation to matters within his expertise and he should not advocate on behalf of one party or the other.

In some adversarial jurisdictions, there are legal rules which provide for the duties of the expert to the court. The main authorities in the UK are the cases of *National Justice Compania Naviera SA v Prudential Assurance Co. Ltd.* ('*The Ikarian Reefer*') (1995) [18] and *R. v Harris* (2006) [19] where the main duties of expert witnesses were outlined as follows:

- The expert must be independent and uninfluenced by litigation;
- he/she must offer independent and unbiased assistance to court;
- any facts or assumptions on which his/her opinion is based must be stated;
- any material facts detracting from his/her opinion must not be omitted from his/her evidence;
- if a matter falls outside the area of expertise of a witness, he/she must say so;
- if insufficient data is available, the expert must indicate that his/her opinion is provisional;
- the expert must, in his/her evidence, set out the full range of available expert opinion in the relevant area, even including opinions that are contrary to his/her own.

In each jurisdiction, these will differ, and sometimes there will be regulatory or professional body considerations too. These duties focus on the witness and not on the evidence.

Juries will not be accustomed (as a judge is) to sitting through long, detailed and technical explanations often in a language they do not understand. Concepts need to be explained for the sake of jurors in an interesting and brief, accurate way. If the jury does not listen and absorb the evidence, the testimony will have been for nothing.

Essentially, most of the jury will not be interested in the details of the science behind the conclusions reached by the scientist. They are interested in what those conclusions are and how they apply to the case they are determining. One tactic to assist in understanding which might enable that to be done effectively is to use analogies to demonstrate the science. For example, it is sometimes useful to refer to everyday objects, and when asked what size is the minimum size of sample that can be analysed, one can describe it as a grain of rice or the volume of a swimming pool etc.

The use of visual aids such as graphs, charts, DVD/video presentations, picture cards, computer simulations (on such simulations see [20]) or even live demonstrations, might liven up the evidence without "dumbing it down". Any unusual method of presentation (anything other than straight oral testimony) should however be brought

to the attention of the court and the other party to the case well in advance, so that approval can be sought from that party and, if necessary, from the court. There may also be logistical considerations involved in setting up technology or equipment in the court, although courts in many jurisdictions are now equipped with impressive technology.

Another possible technique involves the use of evidence arising from 'tailor made' scientific experiments that could be performed especially for the case in question; these may involve effectively reconstructing the incident in question, as has happened in the Scottish criminal courts in *Campbell v HMA* [21].

The presentation of evidence to a non-expert in a mock session is a good way to practice the art of communication to a jury. However, if training is carried out prior to a court case, familiarisation is fine, but the expert must not discuss the case or in any way be 'coached'. In addition, the jury will expect an expert witness to present his evidence in an authoritative manner, without adopting a condescending tone. An expert may have a very good knowledge of his/her subject area, but it is just as important to present that knowledge in a confident and engaging manner. Useful in this context are 'primers' and books which have simplified the background science, containing soil and other ecological evidence types. Effective communication with key audiences is vitally important [22].

17.7 Admissibility of Novel Expert Evidence

Admissibility is the legal concept which refers to the assessment by the court (usually the judge even in a jury case) as to whether or not evidence should be admitted for the fact finder (usually a jury) to assess. When it comes to scientific evidence (especially of a novel nature) the court is concerned with reliability of that evidence, and in particular with the techniques for its collection, storage and interpretation. The issue is that judges have a tendency to be worried about the 'mystic infallibility' [23] of experts. Although the wording differs from one jurisdiction to another, the overall thrust of admissibility of novel evidence is similar. Some of the main considerations for whether the courts may permit a scientific technique to be examined in court are as follows:

- necessity of evidence (can the jury reach conclusions on the issue without it?);
- the qualifications of the expert;
- the reliability of the science underlying the conclusions;
- testing of the scientific technique;
- peer review history;
- error rate;
- acceptance within the scientific community;
- is there a mathematical formula, probability statistic, database or some other objective touchstone?

In the US in particular, the courts have demonstrated a willingness to examine in detail each of these factors, and others, during a *voir dire* (a special hearing, including oral testimony, on whether the evidence is to be permitted, held before the final hearing), to come to a final conclusion on admissibility. This can lead to extensive and detailed scrutiny, perhaps lasting several days, or even weeks. While there is variance across jurisdictions in the rigour with which the courts approach admissibility examinations of expert scientific evidence (roughly speaking, the US at the most rigorous end and the UK at the other, with Canada and Australia somewhere in between), it is suggested that it is best practice to assume a fully rigorous examination of the factors above when considering how to defend any proposed expert scientific evidence. After all, even if these factors are not used to argue against admission of the evidence, they will be relevant to the questions of reliability and weight, questions which apply to the assessment of all expert evidence by all decision makers.

The courts have shown willingness to open doors to evidence of all kinds, from any background of knowledge, not just the traditional scientific fields. Recently there has been a dramatic increase in the types of evidence presented by experts in courtrooms. Expert scientific evidence has found a place in courts for a long time, and some forms of expert evidence are commonplace and well established in court, including fingerprint evidence, DNA evidence, ballistics evidence and some forms of physical trace evidence. However, where the evidence involves a new technique, even where it is a variation in the area of an already established field, the courts will apply the same basic tests; in some jurisdictions, they will proceed with extra caution.

Some of the considered new areas of expertise include footwear comparison, ear print identification, CCTV footage facial mapping, hypnosis, voice comparison analysis, hair analysis and psychological autopsy evidence. Given that soil forensic science in many cases uses existing and accepted scientific techniques [3], although applying them to the analysis of soil, there would seem little room for an argument that soil forensic evidence (in a general sense) should not be presented as expert evidence. Of course, whether such evidence should be produced in a particular case is a different question.

In the US, given the rigour of the *Daubert* formula as it applies to any expert evidence [24], there is no sign of a more cautious approach in the case of novel scientific evidence. However, the courts do accept certain forms of evidence which have an established pedigree, such as DNA evidence (*US v Martinez* [25]).

According to Cross and Tapper [26] and the Court of Appeal in *R v Dallagher* [27], the same appears to be the case in the less rigorous jurisdiction of England and Wales.

In Australia, the court in the *Bonython* case [28] indicates:

> If the witness has made use of new or unfamiliar techniques or technology, the court may require to be satisfied that such techniques or technology have a sufficient scientific basis to render results arrived at by that means part of a field of knowledge which is a proper subject of expert evidence.

This signals a more cautious approach in cases where novel evidence is proposed. This was applied again in Australia in R v Murdoch (No. 2) [29] a case where certain DNA evidence was held to have passed the admissibility test. The Bonython court cites earlier examples of new evidence, such as R v McHardie and Danielson [30], a case involving a new mathematical formula used in voice identification.

In Canada, in the landmark case of R. v Mohan [31] the Supreme Court offers the following guidance:

> …expert evidence which advances a novel scientific theory or technique is subjected to special scrutiny to determine whether it meets a basic threshold of reliability and whether it is essential in the sense that the trier of fact will be unable to come to a satisfactory conclusion without the assistance of the expert.

The court in that case approved the approach in an earlier Canadian decision (*R v Melaragni* (1992) [32]), in which additional criteria in this area were set out:

(1) Is the evidence likely to assist the jury in its fact-finding mission, or is it likely to confuse and confound the jury?

(2) Is the jury likely to be overwhelmed by the "mystic infallibility" of the evidence, or will the jury be able to keep an open mind and objectively assess the worth of the evidence?

(3) Will the evidence, if accepted, conclusively prove an essential element of the crime which the defence is contesting, or is it simply a piece of evidence to be incorporated into a larger puzzle?

(4) What degree of reliability has the proposed scientific technique or body of knowledge achieved?

(5) Are there a sufficient number of experts available so that the defence can retain its own expert if desired?

(6) Is the scientific technique or body of knowledge such that it can be independently tested by the defence?

(7) Has the scientific technique destroyed the evidence upon which the conclusions have been based, or has the evidence been preserved for defence analysis if requested?

(8) Are there clear policy or legal grounds which would render the evidence inadmissible despite its probative value?

(9) Will the evidence cause undue delay or result in the needless presentation of cumulative evidence?

This list is not necessarily exhaustive; furthermore, the importance of any one or more of these factors will vary depending upon the particular circumstances of the case.

In the US, there are some commentators who take the view that soil forensic evidence would not currently pass the *Daubert* test [33]. Others form the view that forensic geology evidence is a 'valid source of scientific evidence' and it has been used in the context of a range of criminal charges including hit and run, rape, murder, assault, and in many civil suit contexts too [34]. In a recent court case in Virginia, it was stated that soil evidence did not undergo a formal admissibility challenge within the US court systems until January 2016. The challenge in *State of Kansas v. Kyle Flack*, (2016) [35] involved the admissibility of soil comparisons at the trial, as well as the qualifications of the forensic geologist who conducted the examinations. The forensic geologist was questioned on a range of topics including: (1) the witness's

education and training, experience performing and testifying in forensic soil examination cases, and history of participation in proficiency testing, (2) the accreditation and quality system of the laboratory, (3) the methods used and if these methods were in common use outside of a forensic context, (4) the results of the examination in this specific case, (5) the interpretation of these results, and (6) the forensic report technical review process. The defence challenged the conclusion reached by the forensic geologist and sought the exclusion of the soil evidence at trial. The judge, however, ruled that the soil evidence, as examined and reported, was admissible in the subsequent criminal trial, and placed no limits on the geologist's testimony [36].

17.8 Conclusions

Forensic geoscience provision in the United Kingdom involves aspects of intelligence, investigation, trace evidence and expert witness communication of evidence within the criminal justice system. This can be provided at any, or all of, the following stages from attendance at the crime scene, through sample examination and analysis, data evaluation, report writing and effective communication in court.

Standards are continually improving and being carefully audited and there are a growing number of well represented networks of experts which enable good collaboration and shared good practice internationally, such as the International Union of Geological Sciences-Initiative on Forensic Geology (IUGS-IFG) and the European Network of Forensic Science Institutes-Animal Plant and Soil Traces (ENFSI APST) [37] all offering a range of complementary and accredited skills of great value in assisting police and forensic and legal practitioners across the UK and abroad. It is reassuring that, although innovative developments in the discipline of soil science and forensic geology are being taken up by forensic practitioners and accepted by law enforcement agencies, caution, clarity and integrity remain the key principles in the application of new approaches, in particular, of new analytical methods.

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Chapter 18
End User Commentary on The Application of Forensic Soil Science in Case Work and Legal Considerations

Lida van den Eijkel

This chapter by Dawson, Parratt and Auchie covers the application of soil science in forensic case work and presents a full discussion of the use of soil traces in criminal law enforcement. It describes the legal context one has to be aware of, the many characteristics of soil that can be examined, and the presentation of the obtained results in reports and courts in the UK with its common law and adversarial legal system. This chapter showcases what has been achieved in the development of approaches and methods used in forensic soil science up to the current times. It discusses some anticipated or desired future developments, including outlining the necessary considerations for use of such new approaches in case work. It importantly outlines the main legal aspects, of relevance for any novel approach or new method of analysis being tested and validated for use in case work, as described in this and the other chapters of the book.

As such, Chap. 17 does not describe a(n) individual (emerging) analytical technique, but, rather, it importantly discusses the current and future use of this type of trace and as well as making considerations for the adoption of any new emerging technique in the forensic science.

Therefore, when asked to write a commentary as an "end user", this reading puts one in a reflective mode about soil traces in forensics. After several decades of soil forensics (or somewhat broader: geoforensics), what has been achieved and what may be expected? Just how useful has geoforensics been, and will it be needed more in the future? Are there factors that prohibit the full forensic use of soil traces and if so, can anything be done about that and is it worth the effort? These questions are difficult to fully answer, but some considerations come to mind.

Using soil traces in criminal investigations did not become as self-evident as the examination of many other types of traces. The book that is often seen as the first systematic description of geo- and soil forensics (*Forensic geology* by R. C. Murray and C. F. Tedrow) dates 1975. Now, over 40 years later, in some recent publications

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soil is still being described as something with "forensic potential". So far, soil in the forensic community has not become an obvious addition to the suite of traces to be examined in criminal cases.

To the soil scientist, the forensic potential of soil is clear enough: soil is everywhere and hugely varied from place to place, it is picked up by people and objects and therefore soil easily becomes a trace as in Locard's "every contact leaves a trace". As a trace it contains information related to a (criminal) activity. Traces of soil can help find answers, as the chapter rightly describes, to two important questions: finding an unknown crime scene from soil traces left on objects like tools or clothing when a crime is suspected (most often places of burial of people or items like firearms or bomb components), and determining if traces of soil on evidential objects originate from a particular and known location (such as a crime scene or an *alibi* location). Of these two questions, the former is not often asked, but when such a question arises, it is important to highlight that a soil trace does contain direct information about the very location one is looking for, as is clearly reported by Dawson et al.

The second question (association of a soil trace and a known location), is much more frequently asked. If it can be established that a soil trace was picked up at a crime scene, this can lead to the trace being crucial evidence in a crime. Its role however may also be more modest. From my own experience, a case comes to mind where the question was whether one or two shovels were used to dig the grave in which three murdered people were found buried. For the investigating police, the answer to this question was meaningful to help decide if more people than the one known suspect were involved in the murders. Since the soil on only one of the shovels was similar to the grave soil, the police decided (of course in combination with other intelligence) not to pursue the search for a conspirator. This way the soil helped clarify the crime scenario and soil traces can very frequently do that in many ways.

However, as indeed the chapter shows very well, it takes quite a lot of facilities and expertise to examine soil traces. No one person can ever manage the complete investigation of soil traces from sampling to final interpretation. Soil forensics depends critically on input from forensic practitioners and scientists of different backgrounds, working as a team. It also takes special facilities like GIS (Geographic Information Systems) and relevant soil databases, which need yet more expertise in applying or take long periods to become fully available. This means that the actual examination of soil traces is difficult to practically organise for a single forensic institute or agency without making huge investments. When such investments are not being made, the soil trace will not be used, experience is not gathered, innovative developments will not take place and eventually the soil trace will not increase its importance as evidence and its adoption in forensic case work. This dilemma needs a managerial resolve to keep soil forensics alive and an organisational solution for the proper conducting of soil trace examinations.

Fortunately, to break this cycle, some good initiatives exist. One approach (for example from Australia) is an explicit partnering between forensic practitioners and soil scientists in research institutes. Traces are first evaluated carefully by forensic technicians employing techniques they also use when examining other traces

(mostly spectrometry). If worthwhile, the traces are given to specialist soil scientists in research institutes, who then employ their full range of instrumentation for analysis and apply their specialist knowledge for further examination. Also, in the last decade in for example the UK and Italy, groups of geoscientists or archaeologists together explore the forensic application of their expertise and offer their services to police organisations directly. This is the case in Scotland, where the development of soil forensics has arisen also through a direct partnership between the scientists at the James Hutton Institute, Scottish Police Authority (SPA) and Police Scotland. Another possibility is to do more than just soil trace examination. Within the Netherlands Forensic Institute (NFI) three teams examine soil: in addition to soil traces, soil samples for pollution crime cases are examined, and archaeologists that perform exhumations study soil as the environment of decaying processes to answer questions about, for example, time of death. This broader application of soil science in case work makes certain investments (such as GIS and databases) more justified.

However, insufficient facilities, expertise and awareness of soil as relevant for criminal investigations are not the only factors that still prohibit the full use of soil in forensics. There are still some knowledge gaps that limit the interpretation of the measurement results of soil examinations. It is for example important to have insight in the transference and integrity of a soil trace. Is the soil on a shoe really from one place or is it a mixture that is acquired by walking around as people do? As mentioned in the chapter, it is better to examine lumpy, coherent, aggregates of soil, but they are not always available. Examining the pollen content of the trace might help, since pollen reflects vegetation and it is known which species of plants can be found together or not, so the total assemblage of pollen in a trace can demonstrate that a soil trace is of mixed composition. Of course this can only be done when it is safe to (finally) destroy the trace, since palynology is a destructive method of examination. So unfortunately, often the integrity of a soil trace, especially small ones, is still very unclear. This limited understanding of the trace, prohibits the use of databases that contain information from much bigger samples, and it makes interpretations uncertain.

Also, appropriate methods to interpret measurement results of soil trace examinations still need further development. After the examination, an inference must be made with the measurement results to decide about the provenance or association of the trace. For this, much information is needed, both about the occurrence of different soils and each characteristic in it and of statistical analysis. This is not a simple task, but good developments may come from the use of GIS, that has possibilities for statistical inference built in.

As for the legal aspects of the applications of soil science in forensics, this chapter rightly draws attention to forensic reporting and presentation in court. Dawson et al. focus on the current situation in the UK, that is, the common law and adversarial procedures, “one of the most challenging environments for any expert evidence”. However, from a Dutch perspective, for a country with an inquisitorial system and only professional judges, it is clear that a lot of the legal considerations about forensic contributions to cases given in the chapter are as valid in an inquisitorial as in an adversarial system. Reporting clearly (professional judges are laypersons too), integrity

in work, and scientific robustness are all equally necessary. Moreover, lawyers in an inquisitorial system do not refrain from asking critical questions and can (and do) involve defence experts. Defence lawyers can always ask the judge who oversees the investigative process leading to a trial to involve a defence expert and the request is usually granted. An important difference between the adversarial and inquisitorial system with regards to forensic work is perhaps the fact that in the inquisitorial system much of the challenge is dealt with beforehand and not during the trial in court. Forensic scientists are either registered and evaluated regularly or are appointed by the judge who oversees the investigative process based on qualifications that must be made clear at the beginning of the examinations. Standards and regulations are everywhere. In any case, all forensic work needs to meet the strictest of criteria for good scientific practice. If the work done satisfies this criterion, it should pass adversarial as well as inquisitorial challenge.

It is clear that a small trace of soil takes a big investment in expertise and facilities. But, seeing the soil trace as a valuable addition to the suite of forensic traces, as I do, it is worth the effort to maintain and further develop soil forensics. Necessary for this are at least the simultaneous availability of expertise in soil science and geology, analytical chemistry (both organic and inorganic), biology (of pollen and fungi, plants and animals), facilities with standardised instruments for analysis, GIS, large enough collections of good data on soils, and (not the smallest problem) well developed methods to interpret the measurement results in such a way that appropriate conclusions can be drawn. Within the forensic world it will take a managerial resolve, and (preferably) the establishment of partnerships with academia, research institutes and stakeholders in public prosecution and police organisations, to achieve this and keep soil a valued part of forensics. Moreover it is clear that to achieve this goal good communication between all parties involved is vital and therefore it is a promising development that, as I can observe, this communication is improving and increasing in many places around the world.

Chapter 19
End User Commentary on The Application of Forensic Soil Science in Case Work and Legal Considerations

Patrick Campbell

Over the past 30 years, the investigation of homicide and other serious crimes has become a far more complex, challenging and specialist arena. In that time, strong and enduring relationships have been forged between those working in law enforcement and Forensic Science.

Significant scientific and technological advancements continue to provide far greater opportunities for investigators, resulting in;

- Many serious cases, both current and historic, being brought to a successful (and swift) conclusion
- Little or no challenges being mounted against presented forensic evidence
- Suspects being eliminated more quickly
- Opportunities for miscarriages of justice being reduced due to the integrity of evidence
- Victims, their families and indeed the public at large having greater confidence in the criminal justice system.

The key discipline of Crime Scene Management has developed markedly in recent times. All major investigation crime scenes must be managed professionally and effectively to preserve the integrity of evidence and indeed often acute challenges can be encountered by law enforcement staff and scientists alike when there are multiple locations which require to be secured/examined.

Police Forces have invested heavily in devising and developing training in disciplines such as Crime Scene Management (CSM), Production/Exhibit Handling and Search Advisor. Senior Investigating Officers (SIOs) view these roles as being key to ensuring forensic integrity and ultimately providing the greatest opportunity to identify the person(s) responsible for the crime under investigation.

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The discovery of a victim after a suspected homicide will result in the activation of Major Crime Protocols governed under the College of Policing, Authorised Professional Practice (APP), and the Murder Investigation Manual (2006) (currently under review).

Ultimately, after life has been pronounced extinct, the crime scene will be secured. Inner and outer cordons will be put in place and its sterility will be maintained at all times by attending officers who will be responsible for keeping a Scene Entry Log.

In Scotland, the next formal step in a case of this nature is to arrange a Forensic Strategy Meeting (FSM). This forum will assess and agree on the investigative priorities, challenges, risks and requirements. It will then examine resource requirements, establishing which specialist disciplines and processes will be deployed and how these will be sequenced/phased in partnership. At this stage, consultation often takes place with the National Crime Agency (NCA) to establish whether they can provide any specialist support to develop the investigation.

The membership of a FSM has changed dramatically over the last 5–10 years. On a regular basis, and particularly in cases with external scenes/deposition sites, specialists from a number of external organisations, and also from academia, are invited to participate.

Experts such as Professor Lorna Dawson, a Forensic Soil Scientist from the James Hutton Institute, Aberdeen, as well as Forensic Anthropologists, Archaeologists, Botanists and Entomologists, have provided advice and opinion in a number of major crime investigations throughout Scotland over the past 10 years.

Their input can be invaluable, especially in cases where an identified suspect requires to be linked through trace evidence (e.g. soil) on footwear/clothing/vehicle tyres, to a deposition site in another part of the country.

They have also given evidence at resultant High Court trials, such as HMA v. Angus Sinclair for the 'Worlds End Murders' following the deaths in 1977 of Helen Scott and Christine Eadie.

The use of combined databases in Geographical Information Systems (GIS) can help to bring together soil, geological, land cover information and police data to enable SIOs to prioritise searches on the ground.

In addition, the examination of historical undetected homicides (otherwise known as Cold Cases) has gained momentum over the past 10 years. The significant advances in Forensic Science, particularly around DNA/Fingerprint technology, have assisted such investigations greatly.

It has to be said, however, that utilising the expertise, skills, techniques and databases of forensic soil scientists, and engaging their network across the UK and beyond, is something that was not considered some 25–30 years ago. As a result, the re-examination of clothing/footwear or other more intimate samples, coupled with a review of initial search parameters, may provide SIOs with a range of new investigative opportunities.

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