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Multimodal Molecular Mass Spectrometry Imaging : Development and Applications in Plant Biology and Forensic Toxicology

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UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES

Section des sciences pharmaceutiques
Spectrométrie de masse du vivant

Professeur G. HOPFGARTNER
Docteur E. VARESIO

**Multimodal Molecular Mass
Spectrometry Imaging –
*Development and Applications in
Plant Biology and Forensic Toxicology***

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention sciences
pharmaceutiques

par

Tiffany PORTA
(France)

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**UNIVERSITÉ
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FACULTÉ DES SCIENCES

**Doctorat ès sciences
Mention sciences pharmaceutiques**

Thèse de *Madame Tiffany PORTA*

intitulée :

**" Multimodal Molecular Mass Spectrometry Imaging –
Development and Applications in Plant Biology
and Forensic Toxicology "**

La Faculté des sciences, sur le préavis de Messieurs G. HOPFGARTNER, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), E. VARELIO, docteur et codirecteur de thèse (Section des sciences pharmaceutiques), J.-L. WOLFENDER, professeur ordinaire (Section des sciences pharmaceutiques), M. KARAS, professeur (Goethe Universität Frankfurt, Institute of Pharmaceutical Chemistry, Frankfurt, Germany), et M. STOECKLI, docteur (Novartis Institutes for Biomedical Research, Basel, Switzerland), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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A Mia

A mes Parents

“It is hard to fail, but it is worse never to have tried to succeed”

Theodore Roosevelt (1858 - 1919)

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Preface

This preface explains the choice of the different pictures illustrating the beginning of each chapters of this thesis.

Chap. 1, Part 1 (page 21) is illustrated by the first X-ray image taken by Wilhelm Röntgen's (1845-1923) from the hand of his wife Anna, on December 22th, 1895. The picture Chap. 1, Part 2 (page 55) comes from an article written by Tatiana C. Rohner *et al.* (MALDI mass spectrometric imaging of biological tissue sections. *Mech. Ageing Dev.* **2005**, 126 (1), 177-185), from the group of Markus Stoeckli (NIBR Novartis, Basel) who is one of the leader in MSI. The picture is also the logo of the official MSI website maldi-msi.org, the reference for the MSI community. Picture

Chap. 2 (page 103, <http://aston.chem.purdue.edu/research/ambient-ionization-methods/desi>) shows Zoltan Takats, a pioneer in the development of atmospheric pressure ionization sources (on the picture: Zoltan next to a DESI source).

The photo Chap. 3, Part 1 (page 135) represents the MALDI-QTRAP mass analyzer (i.e. a prototype of the commercialized Flashquant, AB Sciex) used to perform the main of the analysis in the third Chapter. Chap. 3, Part 3 (page 175, <http://www.depts.ttu.edu/chemistry/Faculty/Dauria/>) is illustrated with pictures taken from the webpage of Dr John D'Auria, from the Department of Chemistry and Biochemistry (Texas Tech University, TX), who contributes to the elucidation of the biochemistry and evolution of tropane alkaloid biosynthesis.

The picture illustrating Chap. 4 (page 281) is a picture of the DMS ion mobility cell mounted on a QTRAP 5500 mass analyzer (adapted from AB Sciex website). The figure Chap. 4, Part 2 (page 297, from Fenn, L.S. et al. Characterizing ion mobility-mass spectrometry conformation space for the analysis of complex biological samples, *Anal. Bioanal. Chem.* **2009**, 394, 235) represents a plot of MALDI-IM-MS conformation space obtained for a mixture of model species representing each molecular class (ranging from seven to 17 model species for each class, spanning a range of masses up to 1,500 Da).

A photograph of the Matterhorn (4,478 meters high) illustrates Chapter 5, Part 1 (page 351), which reviews the challenges of quantitative mass spectrometric imaging. The Matterhorn is the famous emblem of the Swiss Alps, on the border between Switzerland and Italy (with courtesy of Dany Spaggiari, LCAP, School of Pharmaceutical Sciences, University of Geneva, Switzerland). The picture, to illustrate this part is given along with a citation of Molière. The combination of the two is self-explanatory.

Pictures illustrating Chap. 3 Part 4a (page 195) and 4b (page 217), Chap. 4 Part 1 (page 283) and 3 (page 311), Chap. 5 Part 2 (page 363) and 3 (page 387), are from my own research articles or production.

Summary of this thesis

The research presented in this thesis was carried out from September 2008 to September 2013 in the Life Science Mass Spectrometry (LSMS) group at the School of Pharmaceutical Sciences of the University of Geneva / Lausanne, in Geneva (Switzerland). The research of the LSMS group began in 2002, and focuses on the development of different mass spectrometry techniques for the qualitative and quantitative analysis of (bio)pharmaceuticals and their metabolites in biological environments. These techniques are employed for bioanalysis, metabolism studies, miniaturization of sample preparation, multi-components analysis, ultra-high throughput and high sensitivity analysis. The development of rapid analytical techniques for the identification and quantification of biological markers in proteomics and metabolomics is also one of the main research areas of the group. The development of molecular imaging based on mass spectrometry approaches and its application to the mapping and quantification of low molecular weight compounds in tissue is one of the interests of the group.

This thesis manuscript is part of the MS imaging project and the main objectives are to develop analytical platforms and their applications in plant biology and forensic toxicology, focused on the analysis of drugs and their metabolites in various biological samples (e.g. plant leaves, hair, and postmortem human tissues).

Chapter 1 is a general introduction and reviews the different imaging techniques that are currently used in biomedical research. *Part 1 – (Bio)medical imaging: from human to molecule* presents the strengths and limitations of imaging techniques currently used in biomedical research. Imaging has played a major role in extending our understanding of human function and physiology for clinical purposes or medical science. Part 1 gives an overview of the main imaging techniques used for the understanding of normal anatomy and physiology but also pathogenic processes, which can be screened, diagnosed and monitored by different methods. These techniques are compared based on the main underlying physical principles that classify those techniques as nuclear or molecular imaging. Furthermore, their capabilities to image living organisms and their potential for biomarker discovery, as part of the drug discovery and development process are explored. In particular, **mass spectrometry imaging (MSI)** has emerged over the last few years in biomedical research as an imaging tool and is increasingly used for biomarker discovery directly from human tissue sections for early diagnosis, prognosis and prediction of cancer. Among other advantages, it doesn't require any specific labeling and thus no prior knowledge of the targeted tissue. MSI allows for the monitoring of molecules involved in biological processes, and is used as a powerful tool to characterize molecular species such as proteins or peptides. *Part 2 – Advanced developments in MSI* highlights the current capabilities and major improvements at each stage of the analytical workflow. This workflow comprises: i) sample preparation; ii) development of new ionization techniques;

iii) MS approaches based on high mass resolution, iv) tandem mass spectrometry or ion mobility; and v) software used for the data processing. These developments have pushed limits in terms of ultra-high spatial resolution, high throughput, and high sensitivity.

Chapter 2 reviews the current status of **secondary ion mass spectrometry** (SIMS) and **matrix-assisted laser desorption/ionization** (MALDI) MS imaging in forensics and plant biology, and highlights the benefits provided by emerging direct surface analysis techniques operated under ambient conditions. This review emphasizes the advantages provided in terms of sample preparation over approaches routinely used in toxicological laboratories, and also how MS-based imaging is gaining interest in the field of plant biology.

Chapter 3 is the main core of this thesis manuscript and focuses on the application of a unique platform based on a matrix-assisted laser desorption/ionization (MALDI) source mounted on a hybrid **triple quadrupole linear ion trap** (QqQLIT) mass spectrometer for mass spectrometry imaging.

Part 1 – Mass spectrometry imaging on a MALDI-QqQLIT platform reviews the basics of the ion formation in the MALDI process. The benefits provided by the atypical hyphenation of MALDI with a triple quadrupole linear ion trap (QTRAP or QqQLIT) mass analyzer for MS imaging of small molecules are also highlighted. Particularly, the strengths and benefits provided by the **selected reaction monitoring** (SRM) mode are emphasized. *Part 2 – Alternative CHCA-based matrices for the analysis of low molecular weight compounds by UV-MALDI-tandem mass spectrometry* explores the choice of a suitable matrix for the analysis of small molecules by MALDI-SRM/MS (adapted from Porta T. *et al.*, *Journal of Mass Spectrometry*, **2011**, 46(2):144-52). *Part 3 – Analysis of tropane alkaloids by MALDI-mass spectrometry imaging in **intact E.coca leaves*** describes the application of the MALDI-QqQLIT platform in chemical ecology to gain information on cocaine synthesis and the evolution of alkaloid formation. *Part 4 – Multimodal analysis of cocaine in single hair samples by combining fast acquisition MALDI-SRM/MS and high-resolution ToF-SIMS imaging* presents one of the most original applications of the platform developed during the PhD research. This approach consists of the monitoring of cocaine consumption in **single intact hair** samples from chronic users by MALDI mass spectrometry imaging. Traditionally, such analysis are performed by liquid chromatography or gas chromatography with MS/MS detection. But these analyses require a complex sample preparation beforehand (i.e. several steps such as pulverization, overnight hair digestion, extractions, derivatization, etc.) and the segmentation of the hair to obtain spatial resolution. As an alternative approach, the present strategy combines a straightforward sample preparation to analyze intact samples and direct analysis by MALDI-MS imaging, where the mass analyzer is operated in the SRM mode. This allows a considerable increase in the throughput of the analysis compared to techniques routinely used (i.e. < 6 minutes per entire intact hair). This strategy is presented as a promising tool for forensics and toxicological screening. This work resulted in a peer-reviewed publication (Porta T. *et al.*, *Analytical Chemistry*, **2011**, 83(11):4266–4272). This

section also introduces preliminary results showing the contribution of ToF-SIMS to high resolution imaging of drugs in hair cross sections. Finally, Part 5 focuses on the *multimodal analysis of drugs of abuse in **postmortem tissue** sections by MALDI-MS imaging and LESA-DMS-MS/MS*.

Chapter 4 presents a new analytical platform based on **liquid extraction surface analysis** (LESA) and **differential ion mobility spectrometry** (DMS), hyphenated for the first time for the investigation of drugs of abuse in tissue sections, including structural isomers, after their separation in the gas-phase and prior to their detection by mass spectrometry. This chapter is divided into three parts: *Part 1 – Automated liquid extraction surface analysis* is a brief review of the principle of surface sampling based on automated liquid extraction, and emphasizes the liquid extraction surface analysis (LESA) mode available on the automated commercialized robot TriVersa NanoMate (Advion Biosciences). *Part 2 – Ion mobility separation methods* presents the growing interest in ion mobility and the benefits provided by this technology in the biosciences. This part reviews the two main operational principles used in ion mobility systems: i) ion mobility spectrometry (IMS), which is a time-of-flight technique based on the measurement of the velocity of ions drifting under the effect of a low electric field (DC); ii) differential ion mobility spectrometry (DMS) or field-asymmetry ion mobility spectrometry (FAIMS), which utilizes different ion mobility behavior in high and low electric fields. *Part 3 – Gas-phase separation of drugs and metabolites using modifier-assisted differential ion mobility spectrometry hyphenated to liquid extraction surface analysis and mass spectrometry* shows the benefits provided by the differential ion mobility device implemented after the automated liquid extraction surface sampling for the investigation of drugs of abuse, including nominally isobaric compounds, for forensics investigations in tissue sections. This work resulted in a peer-reviewed publication (T. Porta *et al.*, *Analytical Chemistry*, **2013**, 85 (24), 11771–117793).

Chapter 5 focuses on quantitative aspects in mass spectrometry imaging and is divided into three parts: *Part I – Challenges for quantitative mass spectrometry imaging (QMSI)* exposes the main challenges inherent in MS-based techniques used for **absolute quantification** of drugs and their metabolites directly from biological tissue sections. These challenges rely on tissue- and analyte- specific ion suppression effects, analyte extraction efficiency (either from tissue sections or intact samples) and diffusion of the analyte standards within the tissue. This section discusses the different strategies developed to address these issues, namely *in-solution*, *on-tissue* and *in-tissue* dilution range strategies. *Part II – Quantification in MALDI-MSI: what can we learn from MALDI-SRM/MS?* discusses the experimental parameters affecting the signal measured and thus the quality of the image generated. This part aims to define what performances can be expected in term of precision for further quantitative analysis by MSI, followed by a SRM-based detection. The experimental parameters considered are the laser frequency, the number of laser shot per pixel, the target plate speed and the MS duty cycle. This part will attempt to respond to the following

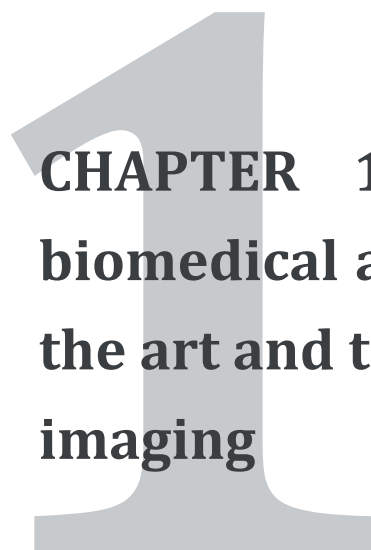
questions: i) how are pixels defined? and ii) what is the minimum number of pixels to be used for quantification? *Part III – Protocol for the generation of suitable standards for quantitative (MALDI) mass spectrometry imaging* proposes a strategy based on a protocol described by Hare *et al.* and widely used for elemental imaging of biological samples by laser ablation-inductively coupled plasma-mass spectrometry. Although the discussion focuses on quantitative MALDI mass spectrometry imaging, the fundamentals of the approach and the issues encountered are relevant to any surface sampling technique.

List of abbreviations

4-desmethyl-OLZ	4-desmethyl-olanzapine
A	Amphetamine
A-d5	Amphetamine-d5
AP fs-LDI	Atmospheric pressure femtosecond laser desorption ionization
APGD	Atmospheric pressure glow discharge
ARG	Autoradiography
BZE	Benzoyllecgonine
BZE-d3	Deuterated benzoyllecgonine
CE	Capillary electrophoresis
cinCOC	Cinnamoylcocaine
COC	Cocaine
COC-d ₃	Deuterated cocaine
CoV	Compensation voltage
CT	Computed tomography
DZ	Diazepam
DZ-d ₅	Diazepam-d ₅
DAPPI	Desorption atmospheric pressure photoionization
DART	Direct analysis in real-time
DMA	Differential mobility analysis
DMS	Differential ion mobility spectrometry
DIOS	Desorption on silicon
DTIMS	Drift time ion mobility spectrometry
EME	Ecgonine methyl ester
EESI	Extractive electrospray ionization
EPI	Enhanced product ion
ESI	Electrospray ionization
FAIMS	Field asymmetric ion mobility spectrometry
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
GALDI	Colloidal graphite assisted LDI
IMS	Ion mobility spectrometry
IR	Infra-red
LA	Laser ablation
LA-ICP	Laser ablation inductively coupled plasma
LAESI	Laser ablation electrospray ionization
LC	Liquid chromatography
LDI	Laser desorption/ionization
LEMS	Laser electrospray mass spectrometry
LESA	Liquid extraction surface analysis

LMJ-SSP	Liquid microjunction surface sampling probe
LZ	Lorazepam
LZ-Glu	Lorazepam glucuronide
M3G/M6G	Morphine-3/6-glucuronide
MA	Methamphetamine
MALDI	Matrix-assisted laser desorption/ionization
MARG	Micro-autoradiography
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MDZ	Midazolam
MOR	Morphine
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSI	Mass spectrometry imaging
nano-DESI	Nanospray desorption electrospray ionization
NCOC	Norcocaine
ND-EESI	Neutral desorption extractive electrospray ionization
NDT	N-desmethyl-tramadol
nES	Nanoelectrospray
nESI	Nanoelectrospray ionization
NIMS	Nanostructure initiator mass spectrometry
NIR	Near-infrared
NorDZ	Nordiazepam
ODT	O-desmethyl-tramadol
OLZ	Olanzapine
PADI	Plasma-assisted desorption ionization
PET	Positron emission tomography
QWBA	Quantitative whole body autoradiography
REIMS	Rapid evaporative ionization MS
SALDI	Surface assisted laser desorption/ionization techniques
SIMS	Secondary ion mass spectrometry
SPECT	Single photon emission computed tomography
SRM	Selected reaction monitoring
S-SSP	Sealing surface sampling probe
SSI	Sonic spray ionization
SV	Separation voltage
SWATH	Sequential window acquisition of all theoretical ions
TOF	Time-of-flight
TLC	Thin layer chromatography

TRMD	Tramadol
TRMD- ¹³ C-d ₃	Tramadol- ¹³ C-d ₃
TWIM	Travelling-wave ion mobility
US	Ultrasound
WBA	Whole body autoradiography

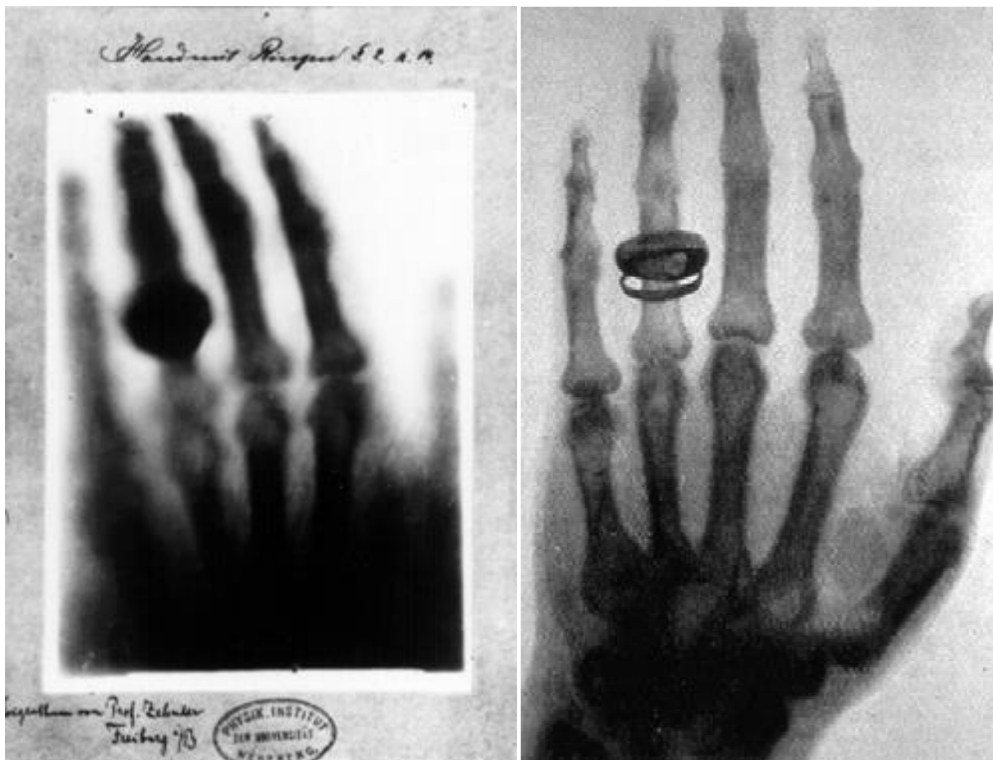


CHAPTER 1. (Molecular) imaging in biomedical and clinical research: state of the art and the role of mass spectrometry imaging

CHAPTER 1.

Imaging in biomedical research and the role of MSI

Part 1 – (Bio)medical imaging: from human to molecule



1.1.1. Introduction

Since the first X-ray image has been taken by W.C. Röntgen in 1895 (Nobel prize for Physiology or Medicine in 1901), the number of medical imaging techniques used to image living organisms has considerably increased. Medical or biomedical imaging refers to the techniques *“using a device to capture images which have medical utility from living humans”*.¹ These imaging techniques played a major role in reinforcing our understanding of human function and physiology to generate structural / mechanical information to assist clinical or medical diagnosis and treatment. But rapidly the question came: can we monitor what is happening at the level of a molecule?

To characterize the dynamic processes that define the state of a biological system, it is essential to establish the relationship between the spatial localization and structure of molecules that are biologically relevant. These molecules or macromolecules are often complex like proteins, peptides, lipids or even DNA. The discovery of the biomarker as a *“characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmaceutical responses to a therapeutic intervention”*² has led to an important area of interest in biomedical research. The objective is to screen, diagnose and monitor changes in organisms due to diseases or response treatments. In the literature, “molecular imaging” often refers to biomedical imaging techniques that rely on the detection of a signal that comes from a specific molecule, sometimes labeled with a radioisotope. This general term is not totally accurate, because the physical principle that governs these techniques is based on the observation of secondary nuclear or molecular phenomena. For instance, although positron emission tomography, single photon emission computed tomography or autoradiography are employed for the study of a specific molecule, they actually rely on the detection of radioactive decay from a specific radiolabeled compound, which is a nuclear phenomenon, and consequently these techniques belong to the family of nuclear imaging techniques.

Molecular imaging techniques in the life sciences aim *“at the investigation of the relation between spatial organization and function of molecules in biological systems”*.³ Techniques based on fluorescence are classified as molecular imaging modalities as they allow indirect detection of a specific molecule emitting light after absorption of electromagnetic radiation. As shown later in this general introduction, fluorescent techniques were used to monitor cellular process in living systems. Imaging approaches based on mass spectrometry, i.e. mass spectrometry imaging (MSI), are the only techniques measuring ionized molecules or atoms. MSI allows for instance for targeted analysis of known compounds and/or discovery of new disease biomarkers.

This first part of this general introduction gives an overview of the main imaging techniques used in (bio)medical research. After a brief introduction of the basic physical principles, the capabilities of these techniques are discussed by addressing the following aspects: is the technique invasive or noninvasive? What kind of information is obtained (anatomic,

structural or functional)? Are exogenous contrast agents or tracers required to improve the contrast within the images? What is the spatial resolution achieved? What are the benefits over the other techniques? Is there a potential toxic exposure on the organism? What are the main applications? Another aspect that will be pointed out is the interest and benefit provided by “multimodality analysis” to link anatomic or structural information with functional information. This was used for instance in oncology for identifying/localizing a mass within a patient body and for determining how the tumor tissue differs from the surrounding healthy tissue. The use of mass spectrometry imaging as a powerful tool for molecular imaging directly from tissue sections has considerably been accelerated over the last decades and is therefore emphasized in this part.

The second part of this first chapter highlights the current capabilities and major improvements at each stage of the analytical workflow that have pushed forward the limits of ultra-high spatial resolution, high throughput, and high sensitivity. This includes developments of sample preparation methods, new ionization techniques, new MS approaches and sophisticated software used for the data processing.

1.1.2. The electromagnetic spectrum

Because most of the medical imaging techniques employ electrical and magnetic fields, or electromagnetic radiation, one cannot talk about biomedical imaging without talking about the electromagnetic spectrum. Figure I- 1 displays the main regions of the electromagnetic spectrum that are of interest for imaging a living organism. Electromagnetic radiation penetrates well into the body at very low (below 200 MHz) or at very high frequencies (above 10^{15} Hz), so that the most useful techniques use either the low- or high-energy window.¹ Also, several regions are out of interest to develop medical imaging techniques. Because tissue water absorbs very strongly above terahertz frequencies, imaging deep tissues is almost impossible in that frequency range, as the radiation cannot pass the outermost layers of the skin and is therefore unable to penetrate the deep organs. Another region where no medical imaging techniques have been developed is the ultraviolet region, for the simple reason that tissues are opaque to the UV light.¹ Obviously, when electromagnetic radiation hits a living organism, it results in side effects that are strongly dependent on the power and the frequency of the radiation, such as local heating when the radiation is absorbed by the cells.

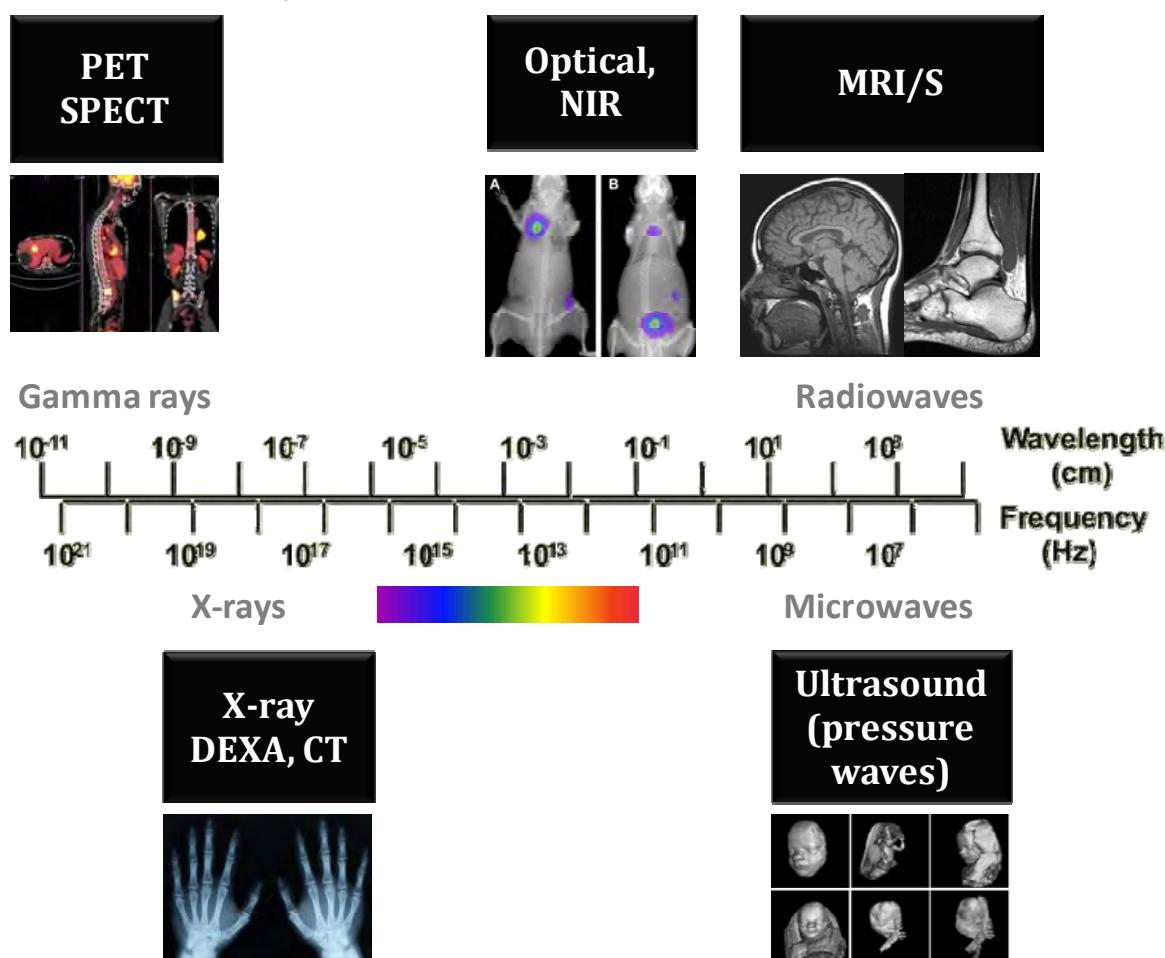


Figure I- 1. Mapping of the main imaging techniques used in biomedical research onto the electromagnetic spectrum (and its equivalent for pressure and sound waves). Adapted from ref.¹

1.1.3. Nonradioactive *in vivo* imaging techniques

1.1.3.1. Magnetic resonance imaging (MRI)

Among the different nuclear techniques, magnetic resonance imaging (MRI) is the mostly used in biomedical imaging for *in vivo* diagnostics. MRI is based on the nuclear magnetic resonance phenomenon to generate microscopic chemical and physical information about molecules. This produces highly defined images of soft tissues and anatomical structures (Figure I- 2a). Unlike X-rays (see §1.1.4.1.), MRI does not expose the patient to ionizing radiation, but uses magnetic fields generated by a magnet surrounding the patient during the MRI exam (Figure I- 2b). Selected applications of imaging modalities based on magnetic resonance are shown Table I- 1.

MRI makes use of strong magnetic fields (i.e. 0.1 to 1.5 Tesla) and radiofrequency pulses (i.e. from 4 MHz to 63 MHz)¹ to measure the proton density. When protons experience a magnetic field, nuclei start to oscillate at a frequency which is dependent on the strength of the magnetic field. If protons are exposed to electromagnetic energy at the frequency of oscillation, they will subsequently absorb energy. When they return to their initial state of equilibrium, they release energy according to two physical processes: the relaxation back to equilibrium of the component of the nuclear magnetization which is i) parallel (characterized by the relaxation time T₁) and ii) perpendicular (relaxation time T₂) to the magnetic field (Figure I- 2c).⁴ This decay generates the signal measured and the contrasts within the images, which are dependent on the microenvironment of the region of interest (i.e. with different protons densities), T₁ and T₂.⁵ To create these contrasts, MRI utilizes protons from water molecules highly abundant but with different concentration in the different tissues. In soft tissue, water concentrations are generally similar and contrasts are thus created by protons from macromolecules (e.g. proteins). Although water and proteins are usually sufficient to produce images with good quality, exogenous contrast agents may be needed. They are typically paramagnetic species with long T₁ relaxation time (e.g. manganese or gadolinium ions), used to increase the relaxation rate of water protons in the body regions where they diffuse.⁵

Compared to other imaging modalities, MRI has a relatively low sensitivity and may require high amount of exogenous contrast agents (§1.1.10. Appendix). The accumulation of contrast agents may lead to the development of severe damages⁶ and the number of intervention per individual should be minimized as much as possible. To limit toxic exposure, the exogenous contrast agents are usually chelated to ensure rapid clearance via kidney and avoid toxicity from the release of free metal ions into the body.¹ In addition, MRI measurements require time, as the return of the nuclei to their equilibrium state does not take place instantaneously, which limits its application for imaging “moving” organs, such as the heart or lungs. In the latter case, breathing motion may hamper the quality of the analysis for instance. Several strategies have been developed to overcome this issue and limit shadowing effects in MR imaging.⁷⁻⁹

Table I- 1. Selected applications of MR imaging and related techniques

Technique	Applications and example of information generated
Magnetic resonance imaging (MRI)	• Functional and morphological imaging (anatomy) • Particularly suited for deep tissues such as brain, neck, spine, limbs, joints and pelvis • Localization and delimitation of tumors in radiotherapy treatment (“monitoring-follow up” tool in the cancer treatment process)¹⁰⁻¹¹ • Measurement of perfusion and flow, by administration of contrast agents (e.g. extracellular fluid agents, intravascular blood pool agents and tissue-specific agents) to enhance blood signal⁶ • Identification of pathologic tissues (e.g. in case of inflammation or neoplasia) by monitoring the endothelial permeability (which increases when a contrast agent administered in selected region leaks into the extra-cellular space)⁵ • Imaging tool for middle cranial fossa, skull base, neural canals and intraspinal contents and is highly sensitive to the presence of c • Highlighting cerebral edema • Evaluation of chronic hemorrhages
Functional magnetic resonance (fMRI)	• In neuroscience research and clinical research • Study of brain activity <i>via</i> the measurement of changes in the blood flow (i.e. generation of blood-oxygen-level-dependent contrasted images¹²) • Used by physicians – assessment of the risk due to brain surgery or similar invasive treatment for a patient • Understanding of functioning of normal, diseased or injured brain / identification of regions linked to critical functions like speaking, moving, sensing, or planning • Used by clinicians – mapping of the brain and detection of the effects of tumors, stroke, head and brain injury, or diseases such as Alzheimer's¹³
Real-time functional MRI (rtfMRI)	• Monitors neurofeedback in response of stimuli in patients suffering from various neurological and psychiatric disorders like Parkinson, schizophrenia, or chronic pain¹⁴

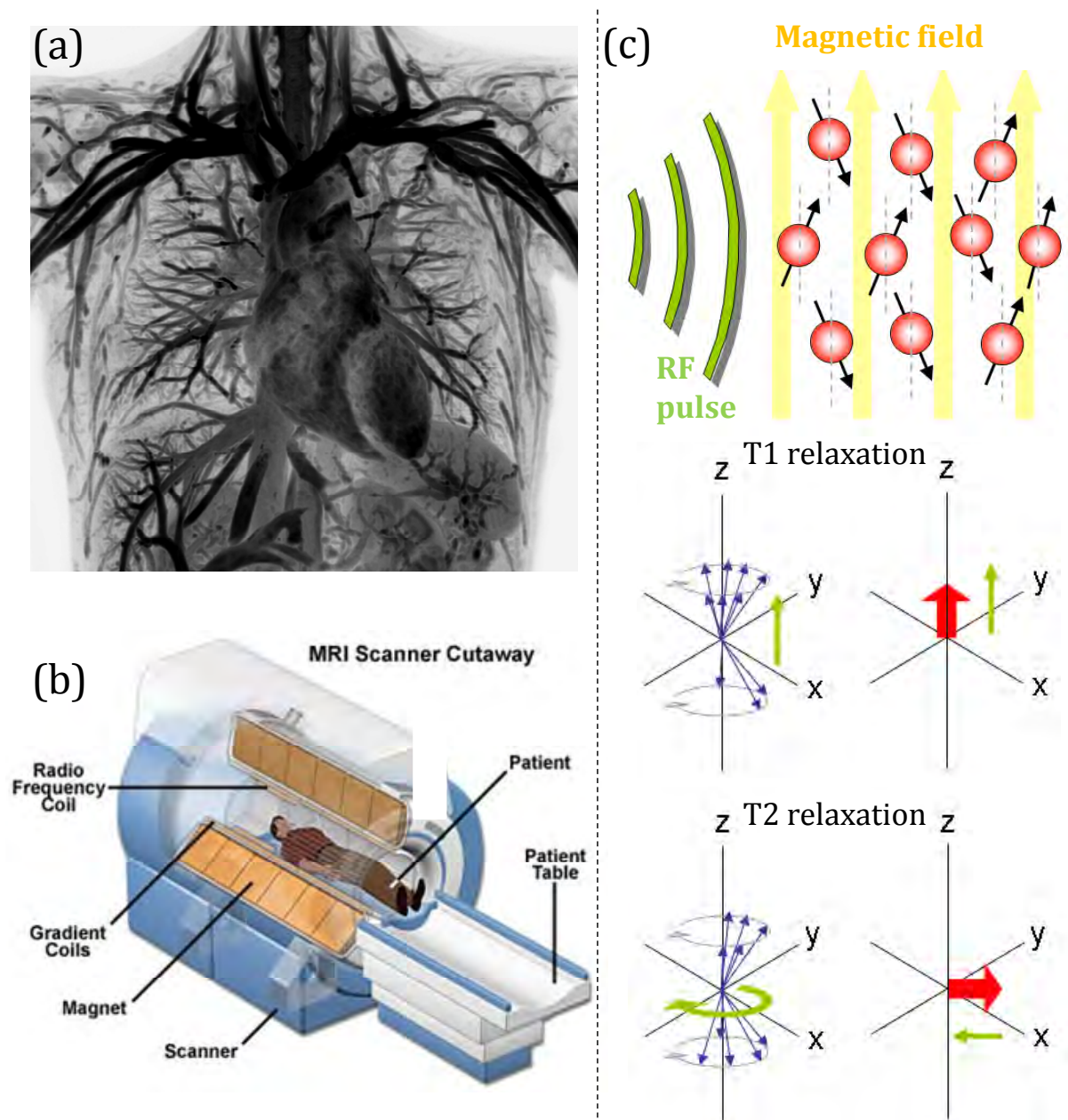


Figure I- 2. Magnetic resonance imaging. (a) High contrast definition image of the thorax by MRI. (b) Representation of the MRI scanner and major components. (c) Schematic representation of the magnetic resonance phenomenon: protons change the direction of magnetization from the Z axis (Longitudinal) to the x-y plane (Transverse), when experiencing polarizing magnetic field and radio frequency at resonance. The overall magnetization (red vector) is equal to the sum of vectors from individual nuclei (blue vectors). Adaptation: (a) from ref.¹¹; (b) from ref.¹⁵ and (c) from ref.¹⁶

1.1.3.2. Ultrasound imaging (sonography)

Ultrasound imaging or sonography is a very popular diagnostic tool relying on high-frequency sound waves to capture contrasted images in *real-time* of internal organs, soft tissues or blood flow inside the body of a patient.¹⁷⁻¹⁸ Ultrasound imaging is inexpensive and very safe (i.e. no ionizing radiation emitted) and is an ideal imaging technique for numerous applications (Table I- 2).

The ultrasounds are produced via a piezoelectric transducer, placed against the skin during the examination. When a sound wave encounters a material with a certain density, part of the sound wave is reflected back to the probe (i.e. echoes) and converted into image by computational processing. The frequency applied usually ranges between 2 to 20 MHz, depending on the target. High frequencies (i.e. 7–18 MHz) are used to image superficial structures (e.g. muscles, tendons, testes, breast, thyroid and parathyroid glands) and neonatal brain. Low frequencies (i.e. 1–6 MHz) penetrate greater in the body and are preferably used to image deeper structures (e.g. liver and kidney).¹⁹ However, high frequencies provide better axial and lateral resolutions than low frequencies.

Contrasts can be enhanced by the use of small gas-filled microbubbles encapsulated in a stabilizing shell (e.g. albumin, galactose, lipids or polymers) (i.e. **contrast-enhanced ultrasound**).²⁰ These microbubbles have a high degree of echogenicity and improve contrasts, after administration in the systemic circulation. The typical diameter of the microbubbles is in the order of a few μm .²¹ The gas core can be composed of air or heavy gases (e.g. perfluorocarbon or nitrogen), less soluble in water and known to last longer in the circulation.²⁰ The contrast is enhanced by the oscillation of the compressible gas bubbles, created by ultrasonic frequencies.

Table I- 2. Selected applications of imaging techniques relying on ultrasounds. From ref.¹⁸

Technique	Applications
Doppler sonography	• Velocity measurement of blood flow in arteries and veins based on the Doppler effect²² • Effective tool for diagnosis of vascular diseases
Fetal ultrasound	• Widely available in hospitals for monitoring of the fetus during pregnancy (see Figure I- 3) • Monitoring of the fetal heart rate (based on the Doppler effect)
Echocardiography	• Reconstruction of cardiovascular motion • Monitoring of heart valves and blood flow¹⁷
Bone sonography	• Diagnosis of osteoporosis
Intraoperative ultrasound (IUOS)	• <i>Real-time</i> guidance or assistance in various surgical procedures to improve intra-operative decision making and surgical procedures²³
Contrast-enhanced ultrasound imaging	• Imaging of regions usually inaccessible like the parenchymal tissues of heart, liver and kidney • Diagnosing and monitoring in cardiology (i.e. myocardial contrast echography, MCE²⁴) • Diagnosing and monitoring cancer and peripheral vascular diseases • Monitoring in drug therapies



Figure I- 3. Prenatal 3D ultrasound image showing baby's face, hand, umbilical cord and knee.

1.1.3.3. Optical imaging techniques

a) Optical techniques for imaging human body

Optical imaging measures the light produced by absorption, scattering, single or two photon emission, fluorescence or bioluminescence of biological or chemical moieties.¹ Typically, optical imaging involves a substance (i.e administered intravenously) that absorbs or fluoresces in the visible / near-infra red region of the electromagnetic spectrum (i.e. wavelengths of 380 to 1'500 nm). The fluorescence emitted by the tagged molecule after illumination of the targeted organ is then captured by specific camera. The excitation light penetrates around 20 mm into the tissue, which limits the applications for imaging the human body to certain regions. Specific instrumentation may be required to access internal regions of the body (like an endoscope, which comprises a rigid or flexible tube, including an optical fiber to illuminate the organ of interest) (see §1.1.10. Appendix).

b) Optical techniques for *in vivo* imaging in rodents

Optical imaging based on bioluminescence and fluorescence (Figure I- 4) is however very popular to image the biomolecular distribution and molecular processes in living laboratory animal (i.e. mice or rats).²⁵ These techniques, although qualified as non-invasive, require genetic modification to obtain the production of light visible through animal tissues. They use specific labels emitting optical photons, with emission wavelengths ranging typically from 480 to 611 nm.²⁶

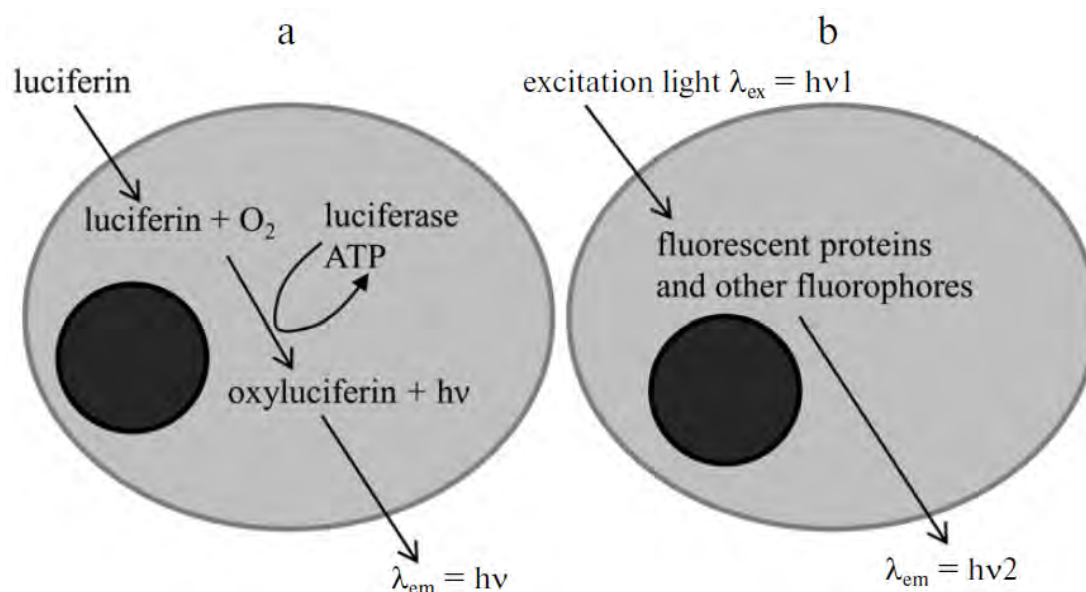


Figure I- 4. Schematic representation of (a) bioluminescence and (b) fluorescence. From ref.²⁶

- **Bioluminescence imaging (BLI)**

BLI (Figure I- 4a) occurs within living organism and relies on the production of light photons from substrate D-luciferin, catalyzed by the enzyme luciferase in the presence of both molecular oxygen and ATP. Bioluminescence tomography (BLT) refers to the technique used to measure the light produced, of which maximum emission is at 560 nm. BLI is thus highly specific and highly sensitive due to the absence of *in vivo* background bioluminescence (see §1.1.10. Appendix). Common applications of BLT are detailed Table 3. The applications of BLI in biology and biomedicine are limited so far due to the cytotoxicity of the substrate D-luciferin. Also, BLI requires the development of suitable carrier that allows simultaneously to deliver the substrate, reduce its cytotoxicity and preserve the bioluminescent property.²⁷

- **Fluorescence imaging (FLI)**

As biological systems constitute a complex assembly of cells with heterogeneous chemistry, characterizing the role each cell plays in the function of these systems is of prime importance.²⁸ In that perspective, fluorescence is playing a fundamental role in biomedical research. Fluorescence is the emission of photons (i.e. light) by a target fluorescent molecule that has been previously excited by an external electromagnetic radiation. Generally, the emitted light has lower energy than the absorbed radiation (i.e. longer wavelength) (Figure I- 4b), except when two photons are absorbed (in this case, the emitted light has shorter wavelength). Although exogenous fluorescent tags can be used, some proteins or small molecules are intrinsically fluorescent. This is for example nicotinamide adenine dinucleotide (NADPH, a metabolite associated with cellular respiration), or the green fluorescent protein (GFP, spontaneously fluoresces upon folding via specific serine-tyrosine-

glycine residues). Fluorescence-based techniques provide high contrast chemical specific images, as fluorescent molecules serve as tags which enable the observation and analysis of the target protein's activity. These proteins can be expressed in cells either alone or as fusion protein, after ligating a fluorescent gene to another gene, in order to provide sub-cellular localization or protein expression patterns. Besides these intrinsically fluorescent molecules, the fluorescence imaging of tissues, cells or subcellular structures can be accomplished by labeling target-specific probe (i.e. antibodies) with a fluorescent chemical moiety. As a result, fluorescent targeted probes, fluorescent proteins and new reporter gene systems have been developed as functional and dynamic markers of *in vitro* and *in vivo* molecular events. Examples of application of FLT and related techniques are given Table 3.

Table I- 3. Selected examples of use of optical imaging in both human and rodent living organisms

Technique	Applications
Fluorescein angiography	• <i>In vivo</i> imaging in human • Visualization of the blood flow of the retina after intravenous injection of a fluorescein solution • Highlighting degenerative changes cause by diabetes • Highlighting high blood flow pressure, inflammation of edema²⁹
Endoscopy	• <i>In vivo</i> imaging in human • Diagnostic and therapeutic tool • Imaging of gastrointestinal tract, respiratory tract, urinary tract...
Bronchoscopy	• <i>In vivo</i> imaging in human • Diagnostic of lung diseases
Bioluminescence imaging (BLI) = bioluminescence tomography (BLT)	• <i>In vivo</i> imaging in rodents • Observation of the <i>real-time in vivo</i> bioluminescence during antibiotic therapy for monitoring infectious disease;³⁰⁻³¹ • Use in cancer research, by the use of bioluminescent cancer cell line³² • Study of neurodegenerative diseases such as Parkinson or Alzheimer's diseases by the use of stem cell and transplantation research³³ • Bioluminescent microcapsules can be used in photodynamic therapy (i.e. a treatment modality for certain malignant tumors, viruses, and some special vascular diseases, requiring light, oxygen, and photoactivating compounds)²⁷
Fluorescence tomography (FLT)	• Whole-body imaging of small animals (possibly in combination with CT or MRI) • <i>Real time</i> monitoring and quantification of biological targets and pathways to give a deeper understanding of the biology underlying disease mechanisms and therapeutic responses³⁴ • Applications in oncology³⁵⁻³⁷ • Characterization of inflammatory disease³⁸, • Characterization of cardiovascular disease³⁹ •

Table I- 3. Continued.

Technique	Applications
Fluorescence resonance energy transfer or Förster resonance energy transfer (FRET)	• Study protein conformation or protein interactions, and also to detect the location and interactions of genes and cellular structures.⁴⁰ • Visualization of the invasion of glioblastoma cells that were implanted into rat brains, after being transfected with FRET-based enzyme activity probes. This study shows the differences in the activities of Rho-family GTPases in cells associated with blood vessels and cells that were not and also highlights the heterogeneity of the cells forming the tumor⁴¹
Fluorescence Lifetime Imaging Microscopy (FLIM)	• Detection of bio-molecular interactions like free and bound NADPH in differentiated and undifferentiated myoblast cells⁴² • Understanding of cellular processes including apoptosis, cancer pathology and also enzyme kinetics
Fluorescence <i>in situ</i> hybridization (FISH)	• Imaging of single protein (molecular level) – but this requires prior knowledge of the sample composition and interferes with the sample's integrity • Examination of the spatial distribution of gene expression, by the use of specific DNA or RNA probes to the cellular targets • Detection of malignant cells⁴³⁻⁴⁶

1.1.4. Ionizing radiation techniques used for *in vivo* imaging

1.1.4.1. Ionizing radiation techniques employing X-rays

Planar X-ray imaging was the first technique used to image the body and is still routinely used as powerful tool diagnostic tool of many disorders (Table I- 4).

X-ray imaging uses a beam of X-rays transmitted through the body. X-rays are composed of photons of which energy is between 100 eV and 100 keV. These photons interact with matter mainly through photoelectric (PE) absorption (i.e. consists of the ejection of an inner-shell electron) and Compton scattering (CS) (i.e. ejection of an outer-shell electron and scattering of a low-energy X-ray).¹ A collimator is used to focus the X-rays beam to ionize only the region of interest. The X-rays that are not absorbed by the body structures are recorded on an X-ray sensitive film, placed below the patient as shown Figure I- 5. The projection of X-ray absorption through the body traduces contrast from electron density, as the absorption of X-ray photons is directly proportional to the atomic number of the absorbing atom.^{1, 47-48} This highlights for instance differences between bones (high electron density thus high absorption, due to abundant calcium [$Z=20$]) and other soft tissues (e.g. low electron density for fatty tissues, with lower absorption due to the high concentration of tryglycerides, containing carbon atoms [$Z=6$]). Exogenous contrast agents may be used and rely on the introduction of dense fluids (i.e. molecules rich in high- Z atoms such as iodine, barium, xenon or thalium).

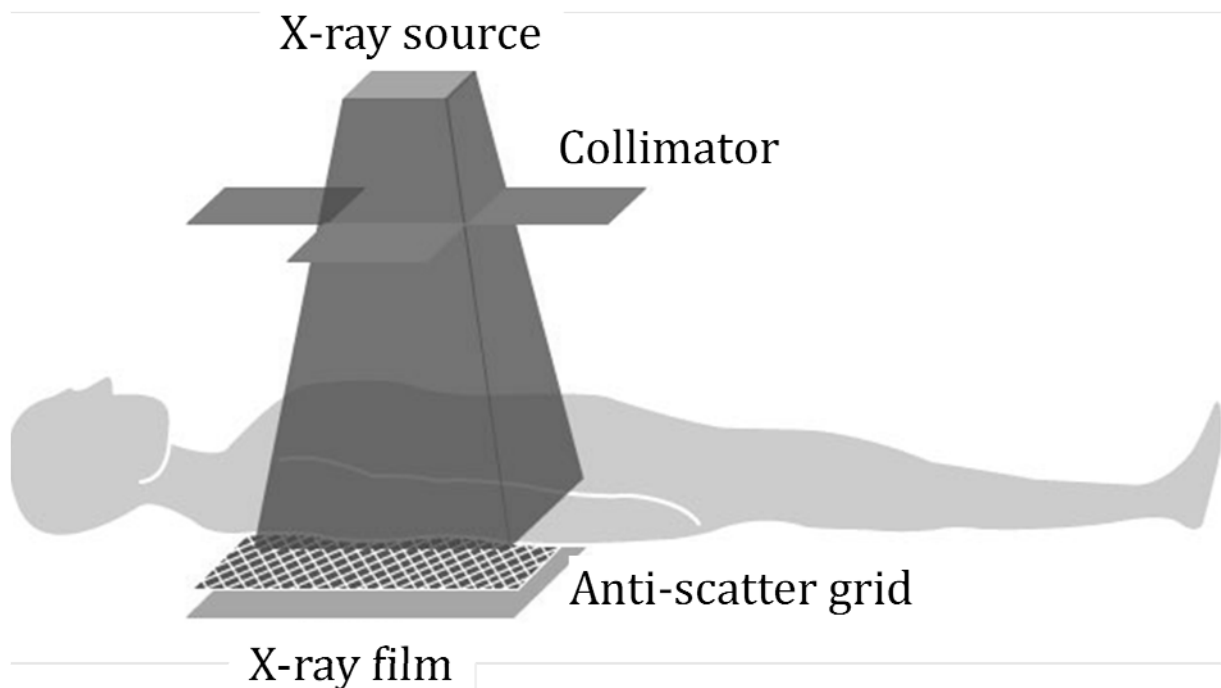


Figure I- 5. Basic experimental setup for X-ray imaging. The anti-scatter grid increases tissue contrast by reducing the number of detected X-rays that have been scattered by tissue. From ref.⁴⁹

The introduction of **computed tomography (CT)** in the 1970s has considerably revolutionized medical imaging and pushed the development of X-ray imaging. A CT scanner comprises an X-ray source mounted on a circular and rotating frame, which also incorporate a detector on the opposite side (Figure I- 6a). Cross-sections of the body are then sequentially imaged when the frame rotates around the patient's body, and 3D images are reconstructed from these "slices". CT scan generates very detailed images and views from different angles (Figure I- 6b). CT-scanners produce images very quickly (i.e. scans completed in the second range⁵⁰) but usually require higher radiation exposure than for planar X-ray exam.

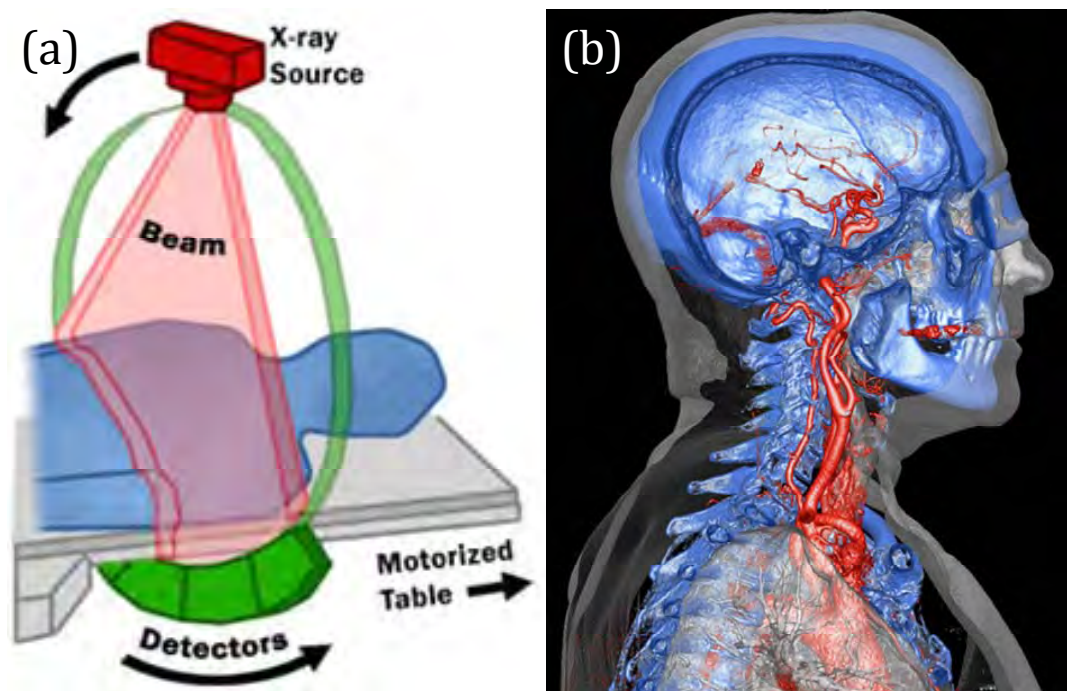


Figure I- 6. Computed tomography (CT). (a) Principle of CT employing an X-ray source and detector unit rotating synchronously around the patient, lying down a moving table. (b) Detailed CT image showing blood vessels and internal organs. (a) from ref.⁵¹ and (b) from ref.⁵⁰

Table I- 4. Use of techniques employing X-rays in biomedical imaging

Technique	Applications
Planar X-rays ⁴⁹	• Diagnosis and treatment of fractures • Diagnosis of osteoarthritis • Monitoring of calcification that can be associated with diseases such as breast tumors or atherosclerotic plaque in the coronary artery¹ • Monitoring lung injuries caused by pneumonia
X-ray CT scan ⁵⁰	• Diagnosis and treatment of fractures • Diagnosis of abdominal diseases • Determination of stage of cancer and monitoring progress • Diagnosis of ischemic stroke • Diagnosis and monitoring of subdural hematoma
Angiography ⁵²	• Examination of arteries, veins and organ • Diagnosis and treatment of blockages or blood vessel problems (thrombosis, pulmonary emboli, vascular malformations)
Fluoroscopy ⁵³ (X-ray fluorescence)	• <i>Real-time</i> examination of the internal structure of the body • Supporting surgeon as a guide for fracture reduction and the placement of metalwork • Guidance for vascular catheter placement interventions⁵⁴⁻⁵⁵
Dual-energy X-ray absorptiometry (DEXA)	• Used to quantitate when several different elements contribute significantly to tissue composition • Measurement of bone mineral density⁵⁶ • Screening of osteoporosis⁵⁶

1.1.4.2. Ionizing radiation techniques using specific radiopharmaceuticals for nuclear imaging

Positron emission tomography (PET), planar nuclear medicine and single photon emission computed tomography (SPECT) are *in vivo* nuclear imaging techniques that require the development of a specific radiopharmaceutical. The selection of a radiopharmaceutical is based on its specificity toward the organ or tissues targeted, or because of its involvement in a physiological function, providing information about metabolism. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) are the two main emission tomography nuclear imaging techniques that exist and differ from the type of radiotracers injected. Although similar in the sense it requires the use of a specific radiopharmaceutical, autoradiography (ARG) is an invasive technique widely used in drug discovery and development, and will be described §1.1.7.

a) Nuclear imaging via positron emission tomography (PET)

Nuclear imaging *via* positron emission tomography, or PET scanning, is one of the most powerful imaging techniques for obtaining functional information and measuring the metabolic activity of an organ, to diagnose and monitor cancer.⁵⁷

The radiopharmaceuticals used for PET imaging are tagged with short decay radiolabel produced in a cyclotron (Table 5). PET measures gamma ray emission produced by positrons, which come from the radiopharmaceutical decay after its administration into the organism. When a positron from the radiotracer is emitted, it rapidly collides with an electron within the tissue, provoking annihilation in a matter-antimatter implosion. From this annihilation result two gamma ray photons, with a precise energy is 511 keV, and which have a trajectory of 180°C from each other. The PET scanner consists of a gamma ray detector, which detects only pairs of photons arriving simultaneously (Figure I- 7a). This allows tracking the distribution and concentration of the radiotracers in real time.⁵⁸ A software program is then used to determine their point of origin and create a three-dimensional image. Unlike SPECT, no collimator is need, and the sensitivity and selectivity of this technique is considerably improved. As a consequence, the amount of radiopharmaceutical administered is considerably reduced (detection down to the picomolar level, see §1.1.10. Appendix).⁵⁷ Typically in oncology, PET studies are performed with a glucose analogue tagged with ¹⁸F (i.e. ¹⁸F-fluorodeoxyglucose, ¹⁸F-FDG), as cancer involves glucose metabolism. Based on the assumption that cancer cells are highly metabolically active because of their rapid rate of reproduction, ¹⁸F-FDG is more actively metabolized in tumor cells than surrounding normal tissue, in the same way than glucose is normally metabolized. It results that the areas with the most intense ¹⁸F-FDG consumption will be emphasized as areas of high metabolic activity, which indicate the presence of cancerous cells that trap ¹⁸F-FDG.⁵⁹ An example is given Figure I- 7.

PET has also shown its usefulness in cardiovascular imaging⁶⁰ for studying myocardial disease (e.g. coronary artery disease)⁶¹ or the cardiac neurotransmission system.⁶²

b) Planar nuclear medicine (= gamma-camera or scintigraphy) and single photon emission computed tomography (SPECT)

Both planar nuclear medicine and single photon emission computed tomography (SPECT), which is the tomographic evolution of planar nuclear medicine, are nuclear imaging techniques using gamma rays emitting radionuclides, of which energy is between 30 and 300 keV. The detection system often requires the use of a collimator, an optical device that modifies the trajectory of the rays emitted to align them in a parallel direction. Because SPECT is less sensitive than PET (i.e. one or two order of magnitude less), higher dose of radiotracer administered is required. However, the radionuclides are more readily available and have longer decay times from hours to few days than those used for PET imaging, i.e. half-lives are of minutes to hours (Table 5).^{58, 65}

SPECT is a valuable technique for functional imaging with various applications in neurology,⁶⁶ oncology (e.g. prostate cancer⁶⁷, thyroid cancer and liver tumors⁶⁸), cardiology⁶⁰ and particularly ischemic heart disease,⁶⁹ endocrinology,⁷⁰ infection and characterize the inflammation in the musculoskeletal system.⁷¹

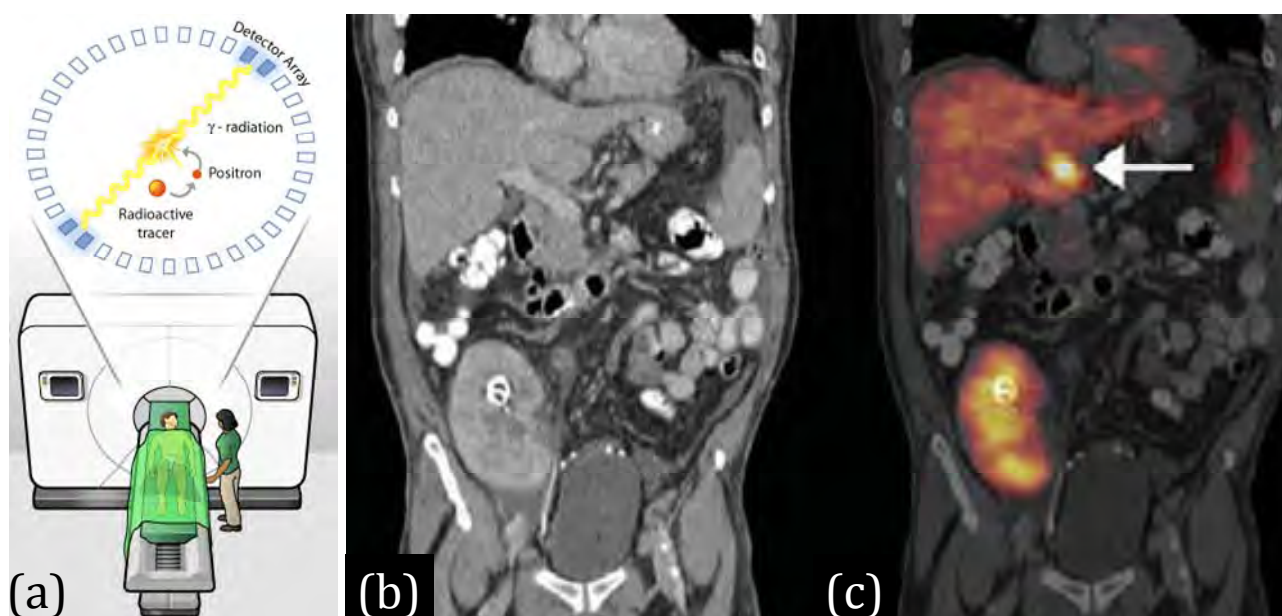


Figure I- 7. Positron emission tomography (PET). (a) Schematic representation of the PET process. (b,c) Example of discordant finding. (c) Abdomen and (c) FDG PET fused with the same CT scan. Arrow indicates the histologically confirmed focal lesion with high uptake of FDG, whereas the CT scan does not show any abnormalities at the site of high FDG uptake. The high uptake in the allograft, including the kidney calices and pyelum, is physiological, as is the modest uptake in liver and spleen. (a) from ref.⁶³ and (b,c) from ref.⁶⁴

Table I- 5. Characteristics of radiopharmaceuticals used for nuclear imaging. ^{99m}Tc = Technetium-99m.

Technique	Type of emission	Radiolabel	Half-life
PET	Gamma-ray emission produced by positrons	¹¹ C	20 min
		¹³ N	10 min
		¹⁵ O	2 min
		¹⁸ F	110 min
SPECT	Gamma-ray	^{99m} Tc	6 hours
		¹¹¹ In	8 days
		¹²³ I	13.2 hours
ARG	Beta-ray emission	¹⁴ C	5 730 years
		³ H	12,3 years
		¹²⁵ I	59 days
		³⁵ S	87.4 days

1.1.5. Multimodality imaging: linking anatomic and structural information

In recent years, multimodality imaging has emerged as the concept of complementing the strengths of one imaging modality with the strengths of another. Multimodality imaging has been further strengthened by the development of hybrid instruments such as **SPECT-CT**, **PET-CT** or **PET-MR** (also called **nuclear magnetic resonance tomography; MRT**).⁷² Before that, morphologic information and functional information were acquired separately in two distinct devices, at different time points, and the main issue was the coregistration of the images. Multimodality imaging is particularly of interest to link anatomic or structural information (usually given by X-ray CT or MR) with functional information (provided by SPECT or PET). Multimodality imaging has found many applications in several areas of biomedical research such as oncology, neurology and cardiology and has been extended to clinical research.⁷³ **MRI** and **optical imaging** such as fluorescence microscopy have also been proven of utmost relevance in drug discovery⁷⁴ and in other areas such as oncology.⁷⁵ **Ultrasound-modulated optical tomography (UOT)** is a hybrid technique which combines advantages of ultrasonic resolution and optical contrasts that allows detection of physiological functions and abnormalities, including potentially early cancer detection. Among the several advantages provided by multimodality imaging, the increase of the spatial resolution and the speed of acquisition to reconstruct internal 3-D structures are among the most important ones. One of the major benefits for a patient suffering from cancer is a better localization of a tumor by the oncologists during surgery, who can treat the cancerous tissues. This limits the side effects during ablation such as damage caused to the healthy tissues surrounding the tumor. As a result, SPECT and PET are often used to

improve diagnostic confidence for radiologists and nuclear medicine physicians and either both can be used in combination with X-ray CT or MRI. This allows for the linkage of anatomical imaging and precise localization of the radiopharmaceuticals administered.⁷³

1.1.6. Imaging in the drug discovery and development process

Although biomedical imaging in humans is mainly employed for screening, diagnosis and monitoring a patient's response to treatment, it is also widely used in clinical research to assist the development of therapeutic drugs in humans. Because imaging has the ability to study various biological and chemical processes, imaging technologies have become an integral part of the drug-discovery and development program and are commonly used in following disease processes and drug action in both preclinical and clinical stages.^{74, 76}

In the past, basic science research has been limited to *in vitro* studies of cellular processes and *ex vivo* tissue examination from suitable animal models of disease. Recent techniques have been developed in the last three decades to allow the imaging of small laboratory animals.⁷⁷⁻⁷⁸ Like for human imaging, these techniques use X-rays, radiopharmaceutical emissions, magnetic resonance signals, sound waves, optical fluorescence and bioluminescence. As a result, in recent years, the tendency has been directed toward the miniaturization of instrumentation to study animals, playing a major role in pre-clinical imaging in improving our understanding of biological behavior and in facilitating the development of treatments. Thus, **micro-ultrasound**, **micro-SPECT**, **micro-PET** and small-bore MR (**micro-MRI**) have emerged and hybrid instruments such as PET/SPECT/CT and PET/MRI⁷⁹⁻⁸⁰ have become available, improving the limitations of separate image acquisitions. Koba *et al.*⁷⁸ point out the major advantages of imaging living animals over other biomedical investigations: i) imaging living organisms supports the study with collection of data in real-time, and thus the number of animals sacrificed can be considerably reduced; ii) diagnostic and therapeutic agents can be developed and tested on nearly identical molecular synthesis platforms and the imaging and quantification of these agents can directly identify their biodistribution, pharmacodynamics, and kinetics, thus providing a uniquely straightforward translational paradigm; iii) by enabling longitudinal studies, imaging of living animals allows continuous, dynamic, and sometimes nearly instantaneous identification/quantification of disease progression and, in many cases, treatment.

"Micro-imaging" usually uses higher frequencies or higher radiation doses than human imaging to increase the spatial resolution. For instance, ultrasound imaging in small animals, which enables *real-time* studies, use higher frequencies of 40 MHz *versus* 1-10 MHz and the spatial and time resolutions are of < 50 μm and < 10 ms, respectively. Micro-MRI increases the spatial resolution down to 50 μm for rodent imaging⁸¹ with very high magnetic fields (i.e. up to 7.0 T for animal studies, while magnetic fields of 3 T (126 MHz) are used for

routine clinical for instance⁵⁷). MRI imaging may require long-time measurement (i.e. up to 1 h) depending on the spatial resolution required⁸¹ and remains highly expensive compared to other modalities. Micro-CT provides cross-sectional anatomical imaging with resolution of 50-100 μm , and even down to 20 μm . However, micro-CT requires the animals absorb higher radiation dose and must be reserved for terminal experiments, as the animals have to be sacrificed at the end of these experiments. Resolution for micro-PET and micro-SPECT depends on the particularities of instrument specifications, but usually resolutions down to 1 mm can be achieved with modern micro-PET, and even less than 1.0 mm resolution can be achieved in certain SPECT configurations.⁷⁸

Complementary to functional MRI described above, **pharmacological MRI** (phMRI) is used in animal laboratories for assaying brain activity after drugs are administered, to check how much of a drug can penetrate the blood–brain barrier and to collect dose *versus* effect information of the medication.^{12, 82-84} PhMRI has been used to evaluate response to treatment, such as the ones developed to treat headache and pain⁸⁵ and neurodegenerative diseases such as schizophrenia.⁸⁶

1.1.7. Autoradiography in drug discovery and development: WBA and MARG

Autoradiography (ARG) was described for the first time in 1867⁸⁷ and became a reliable technique for investigating the localization of compounds in biological specimen from 1953-1954.⁸⁸⁻⁹⁰ Although ARG is described as *“the first molecular imaging technique used for the localization of radioactivity in tissue and pharmacokinetics of new radiolabeled chemical entities in biological specimens”*,⁹¹⁻⁹² ARG actually relies on a nuclear phenomenon. In the same way than SPECT or PET, ARG uses specific radiopharmaceuticals labeled with an isotope having low beta radioactivity (see Table I- 5) and without affecting the chemical properties and/or its intended effects. The main difficulty of the technique is to label the drug at a position that will be kept by the resulting metabolism products, so that the overall activity can be measured. No one can differentiate the parent drug from its metabolites and/or potential degradation products, and a prior knowledge of the drug metabolism is required to accurately interpret the data.⁹¹ Unlike SPECT or PET, ARG is a destructive technique and requires the sacrifice of animals.

ARG is used in pharmaceutical research to image molecule distributions at the scale of a single cell (i.e. micro-autoradiography), an organ or a whole-body of laboratory animals such as rats, mice, rabbits, dogs and even monkeys (i.e. macro-autoradiography). The different terminologies used in the literature to describe ARG techniques are depicted in Table I- 6.

Table I- 6. Terminology used in the literature to describe ARG techniques. From ref.⁹²

Name of the technique	Definition
Autoradiography (ARG)	Any technique whereby radioactivity from within a sample is imaged using a photographic emulsion*
Macro-autoradiography	Any technique where radioactivity is imaged in large samples (e.g. whole-bodies, organs systems, organs of large animals such as cows) using a photographic emulsion*
Whole-body autoradiography (WBA)	A qualitative photographic technique whereby radioactivity administered to an animal is imaged in all tissues of the body using whole-body sections obtained from intact snap-frozen animal carcasses
Whole-body autoradioluminography (WBAL) or quantitative whole-body autoradiography (QWBA)	A qualitative and quantitative digital imaging technique whereby radioactivity administered to an animal is imaged in all tissues of the body using whole-body sections exposed to a phosphor image plate**
Micro-autoradiography (MARG)	A histological technique where radioactivity from within a small cellular, tissue, or organ sample is imaged using a photographic emulsion* and the results are viewed under a microscope

* a *nuclear photographic emulsion* is a fine suspension of insoluble light-sensitive crystals in a fluid (usually gelatin in solution). The passage of charged subatomic particles (e.g. decay of short-lived radiopharmaceuticals) is recorded in the emulsion in the same way that ordinary black and white photographic film records a picture. After photographic developing, a permanent record of the paths of the charged particles remains and may be observed through a microscope;⁹³

** *phosphorimaging* is a form of solid-state liquid scintillation where radioactive material can be both localized and quantified. It measures the light emitted from excited electrons returning to their ground state.⁹⁴

1.1.7.1. Whole body autoradiography (WBA) or quantitative whole body autoradiography (QWBA)

QWBA is particularly suited to quantitatively image the distribution of a radiolabeled pharmaceutical administered to a laboratory animal. A whole-body tissue sections consists in 35-40 different types of tissues.⁹¹ Unlike other tissue analysis approaches such as organ dissection / homogenization / liquid scintillation assay (LSC), QWBA allows for characterizing *in situ* pharmaceutical entities during drug discovery and development process, and for determining the spatial distribution of a drug and its metabolites.

After the sacrifice of the animal by euthanasia or deep anesthesia,⁹² the entire carcass is snap-frozen in a dry-ice-hexane bath and then freeze embedded in a block of embedding media (usually 1-5% carboxymethylcellulose, CMC). Finally the block is cryosectioned in a large cryomicrotome into slices that are collected onto adhesive tape.⁹² By using long-lived ¹⁴C and ³H isotopes as label, QWBA allows for the tracking of drugs, but also their metabolites, over a long period of time, which would not be possible with PET or SPECT. However, some studies report the use of ¹⁸F (half-life of 110 min) radiolabeled compounds

(i.e. ^{18}F -FET) to detect tumors in non-neoplastic inflammatory diseases,⁹⁵ in the same way that PET uses the ^{18}F -FDG analog.

In combination with phosphor imaging technology, QWBA is to date the only tool that allows for the absolute quantification of drugs/metabolites within a whole body tissue section. Unlike other direct surface analysis techniques (e.g. mass spectrometry-based imaging employing MALDI as ionization source), it does not suffer from any suppression effect coming from blood or different types of tissues. Calibration curves are usually made from spiked whole blood that are then embedded in a polymer to reconstruct a block and cut into sections.⁹⁶ The only condition for performing quantification is the thickness of the standards must be exactly the same than the thickness of the tissue sections that are usually of 20–50 μm . This allows for collecting whole-body tissue section onto adhesive tape and also to generate enough radioactivity. For absolute quantification, it is assumed that all tissues analyzed have density and quench characteristics similar to whole blood.⁹⁶⁻⁹⁹

One of the major limitations of the technique is that both the parent drugs and their metabolites, so that the radioactivity measured is actually an overall activity and one cannot tell where the parent drugs or the metabolites are specifically localized within the whole-body tissue sections. Most of the time, experimental designs consist in only 1 animal per time point and more time points are used to determine accurately pharmacokinetics in every compartment of the body; or less time points less animals (i.e. 3-4) per time point compared to other techniques.^{92, 100}

QWBA has rapidly grown as a powerful tool in drug discovery to localize large and small molecules in absorption, distribution, metabolism and excretion (ADME) and pharmacokinetics studies, to determine potential toxicological issues, and also to study drug-drug interactions, tumor, and brain and cerebrospinal fluid penetration and many others.^{92, 96, 100-106} To illustrate, QWBA was used to demonstrate the preferential uptake of the non-steroid anti-inflammatory drug diclofenac into inflamed tissues after a single oral dose in rats (Figure I- 8).¹⁰⁰ Finally, the slow deep-freezing step does not prevent cellular damage and thus WBA is unfortunately not compatible with histology. This is where micro-autoradiography is needed.

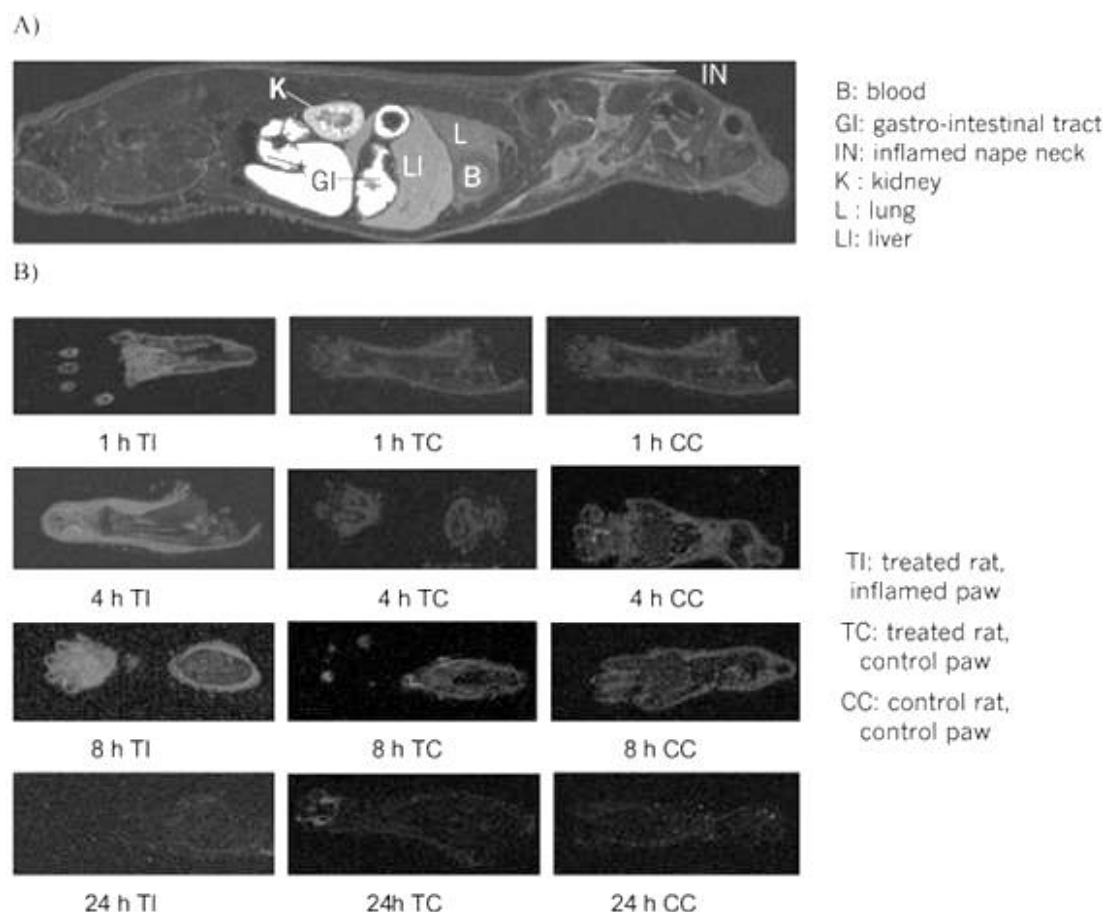


Figure I- 8. Selected whole-body autoradiograms of treated rats after 4 h in the whole animal (A), and in the injected footpads of the treated and the control animal (B) after a 2 mg/kg oral dose of $[^{14}\text{C}]$ diclofenac sodium. The white areas correspond to the high radioactivity concentrations. From ref.¹⁰⁰

1.1.7.2. Micro-autoradiography (MARG)

MARG is a high-resolution tool to investigate spatial localization of radiolabeled drugs in tissue and at a cellular scale. The spatial resolution achieved with MARG is down to 10 μm . For that purpose, preparation of animals slightly differs from the protocol described previously for QWBA: i) after dosing, the animal is sacrificed by exsanguination, ii) tissues are dissected and snap frozen in isopentane, which is cooled in liquid nitrogen (i.e. cooling method “softer” than dry-ice-hexane bath); iii) individual tissues are then cryosectioned at the optimal cutting temperature for a given tissue/organ into 4–5- μm -thick sections; iv) sections are thaw-mounted on dry glass microscope slides pre-coated with nuclear photographic emulsion; and finally v) the slides are placed into a light-tight box with desiccant and allowed to expose for an appropriate amount of time.⁹²

After the MARG image has been taken, the tissue sections can be stained following conventional histological staining for further histological determination, including immunostaining to provide additional insight regarding *in situ* co-localization of drug-derived radioactivity into known cell types or receptor. MARG has predictive value for specific drug

targeting and found its main applications in drug metabolism, toxicology, pharmacology and molecular biology.^{92, 107}

To date, the ability of MARG for absolute quantification has not been proven, partly due to the uniformity of tissue and emulsion thicknesses, and the difficulty in collecting tissue sections under darkroom conditions. In addition, while the exposure time to get images with good quality is of up to 15 days with QWBA, it takes from weeks up to several months to obtain optimal results with MARG.

1.1.8. Molecular imaging based on mass spectrometry

1.1.8.1. The growing interest for MSI in biomedical and biological research

Mass spectrometry imaging (MSI) has improved over the years and demonstrated its great potential for biomedical diagnostics and research.¹⁰⁸⁻¹¹⁰ Particularly, MSI is promising for protein analysis directly from intact biological tissues.¹¹¹ It shows, for instance, the correlation between the relative abundance of proteins and peptides and their spatial distribution within a tissue section.¹¹²⁻¹¹⁶ This helps to better understand and characterize the biological environment that surrounds us, to explain diseases or metabolic disorders. MSI is also used as a tool to support drug discovery and development and move towards personalized medicine.¹¹⁰

Mass spectrometry “possesses the unique capability to measure masses of individual molecules that have been electrically charged. Mass spectrometry is a powerful technique that is used to identify unknown compounds, to quantify known materials, and to elucidate the structure and chemical properties of molecules.”¹¹⁷ Contrary to other imaging techniques such as positron emission tomography (PET) or whole body autoradiography (WBA), mass spectrometry-based imaging is a label-free technique and enables to monitor thousands of compounds in a single experiment. After desorption of molecules at the surface of a tissue section, the molecules enter the ionization source where they are converted into ions. The ionized species are then analyzed through the mass analyzer according to their mass-to-charge ratio (m/z). Mass spectra are acquired at each position of the sample and a 2D map can be reconstructed for each ion, where one mass spectrum represents one pixel in the 2D image (Figure I- 9). Therefore, the relative abundance of thousands of molecular species can be correlated to their spatial location.

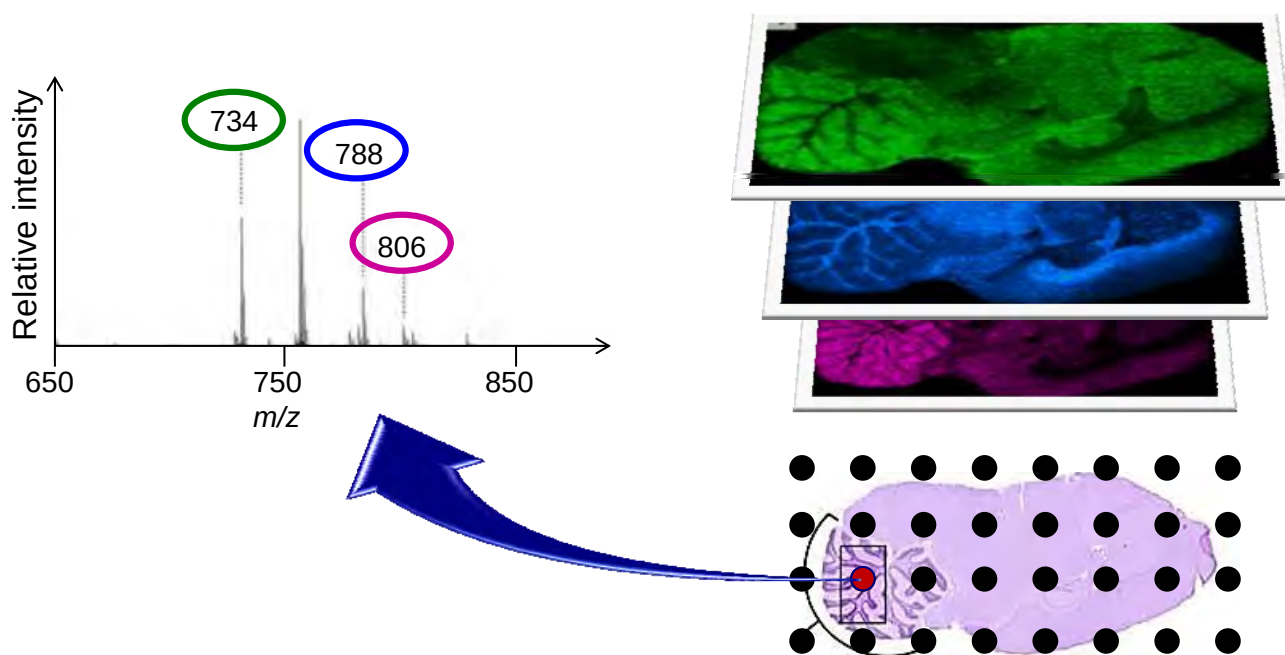


Figure I- 9. Correlating specific molecular information and spatial localization by mass spectrometry imaging (MSI). On the left part, a representative MS spectrum is shown, acquired from a sagittal rat brain tissue section. The MS images shown on the right part show specific images of three different lipid species with m/z 734 (green), m/z 788 (blue) and m/z 806 (purple). Adapted from ref.¹¹⁸

Extensive efforts in instrumental developments were achieved to push the limits in terms of ultra-high spatial resolution, high throughput, and high sensitivity. The resulting performances to date enable to i) obtain molecular information down to the cellular level (e.g. to distinguish healthy from cancer cells); ii) fast generation, acquisition and processing of data and iii) detect both targeted and untargeted compounds even at low abundances.

1.1.8.2. Imaging at cellular scale

A tumor is a highly complex system at the cellular level, composed of distinguished cell types at various development stages. Due to the complexity and rapid change of protein patterns in differentiating cells, new molecular tools are needed to identify not only the changes in protein expression, but also the specific proteins involved. High resolution imaging combined with molecular specificity would thus be extraordinarily beneficial for a better understanding of the construction of a tumor system. For instance, this would allow for the identification of specific regions such as the invasion front, which differs functionally from other regions, and hypoxic regions inside tumors may be metabolically different from the rest of the tumor.¹¹⁹ Developments of sampling techniques have enabled for imaging at cellular length scales.¹²⁰ Advances in ion probe technologies have increased the dynamic range and sensitivity of analyte detection by SIMS, allowing 2- and 3-dimensional localization of analytes in a variety of cells.¹²¹⁻¹²³

Targeted multiplex imaging mass spectrometry utilizes several different specific antibodies containing laser cleavable mass tags bound to specific antigens, which allows for detecting multiple antigens in a single tissue section and imaging of targeted single cells. This technology is applied to visualize the co-expression of synaptophysin and two major hormones peptides, insulin and somatostatin, in duplex assays in beta and delta cells contained in a human pancreatic islet.¹²⁴

Laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), commonly used to trace and ultratrace analysis of multielements, has recently emerge has a promising tool in the MS imaging area to study metals and metalloproteins involved in several diseases, such as Alzheimer's disease.^{120, 125-130} LA-ICP-MS offers spatial resolutions down to 10 to 200 μm , enabling single cell analysis.

1.1.8.3. MSI Applied to biomedical research and identification of protein biomarkers

One area of intense interest is the use of profiling and imaging MS to define the molecular phenotypes expressed in cancer. In proteomics, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is being more frequently used for the discovery of biomarkers. Two strategies are employed for protein identification: top down and bottom up. Top down strategy relies on intact protein analysis, where tandem mass spectrometry (MS/MS) fragmentation is usually performed for identification.¹³¹⁻¹³² Bottom up approach consists in *in situ* enzymatic digestion.¹³³⁻¹³⁸ The typical analytical workflow for on-tissue protein digestion is the following: after a sample clean-up step (see Part 2, §1.2.2.2), an enzyme (e.g. trypsin) is deposited by micro-spotting on a specific area of the tissue or on a very small selected area (microdigestion) and incubation occurs overnight. After drying the tissue section, the MALDI matrix (i.e. CHCA) is applied, followed by MALDI-MSI analysis. This generates peptides of 400 to 3500 Da, which is also a mass range where instrumental sensitivity is higher than that for intact proteins.¹³⁴ Peptides fragments and proteins can be thus identified by tandem mass spectrometry (e.g. by collision induced dissociation) and peptide mass fingerprint (PMF).¹³⁸ *In situ* tryptic digestion protocols were developed either from frozen¹³⁹ or formalin-fixed paraffin embedded (FFPE) tissue sections.^{137, 139-142} MSI is also often used in combination with hematoxylin and eosin staining and immunohistochemistry to validate the results.

Based on the differential expression of proteins from one tissue to another, MSI has thus emerged as a precious tool in histology to differentiate healthy from tumorous tissues in human biopsies and animal models through the investigation of the structure, function and dynamics of proteins and their organization³ in lung,¹⁴³⁻¹⁴⁶ colorectal,¹⁴⁷ breast,¹⁴⁸⁻¹⁵⁰ brain,¹⁵¹⁻¹⁵² skin,¹⁵³⁻¹⁵⁸ prostate,¹⁵⁹⁻¹⁶¹ pancreatic¹⁶²⁻¹⁶⁴ and ovarian cancers.^{137, 165-166} Since the expression profiles of numerous proteins can be obtained in a single experiment and don't require specifically labeled antibodies, MSI is expected to considerably increase the throughput for discovery of new protein markers.¹¹¹ MSI has also been applied to

neurodegenerative diseases such as schizophrenia,¹⁶⁷⁻¹⁶⁹ Parkinson^{129, 170-174} or Alzheimer's¹⁷⁵. In the later, MALDI-MSI particularly allows to image the distribution of the β -amyloid peptide in brain¹⁷⁶ or whole-body mice sections.¹⁷⁷ The β -amyloid peptide is the main component of amyloid plaques responsible for the Alzheimer's disease. To illustrate Figure I- 10 displays images of selected proteins, showing an actively growing tumor.

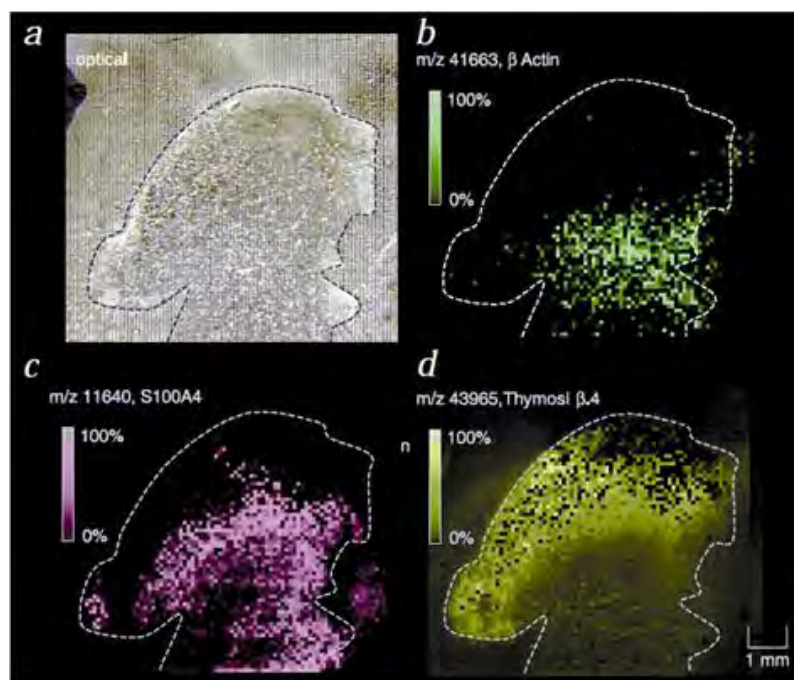


Figure I- 10. Selected protein images from a glioblastoma section showing an actively growing tumor. *a*, Human glioblastoma slice mounted on a metal plate, coated with a matrix. *b-d*, Mass spectrometric images of proteins showing high concentration in the proliferating area of the tumor (*d*) and other proteins present specifically in the ischemic and necrotic areas (*b* and *c*). From ref.¹⁷⁸

MSI has also been a success in many other applications such as in ophthalmology to study glaucoma and/or lipid distributions in the ocular lens,¹⁷⁹⁻¹⁸⁴ kidney disease,¹⁸⁵ musculoskeletal disease (e.g. rheumatoid arthritis¹⁸⁶), osteoarthritis in human cartilage,¹⁸⁷⁻¹⁸⁸ nephropathology prescreening (i.e. understanding early stage renal malfunctions with regard to the glomerular filtration processes).¹⁴¹ Recently, kinetic mass spectrometry imaging (kMSI) has been developed for imaging metabolic turnovers (i.e. continual exchange of cellular components by degradation and synthesis), based on the *in vivo* incorporation of stable isotope-labeled precursors into intact, non-fragmented molecules.¹⁸⁹

1.1.8.4. The use of MSI in pharmaceutical research

MSI is of a great interest for pharmaceutical research and has been extended to the analysis of low molecular weight compounds for drug and metabolism distribution studies.^{143, 167, 190-193} It allows for the spatial localization of an administered pharmaceutical compound (i.e. the “parent drug”) correlated to potential pharmaceutical or toxicological effects (e.g.

therapeutic response, given by local protein expression changes). As an example, Figure I-11 displays the spatial distribution of olanzapine (an antipsychotic drug approved by the Food and Drug administration for the treatment of schizophrenia and bipolar disorder), and two of its metabolites, N-desmethyloanzapine and 2-hydroxymethyl-olanzapine. The differentiation between the parent drug and its metabolites was so far not possible with other techniques such as whole-body autoradiography (WBA), where the overall radioactivity is generated by the tagged parent drugs and its metabolites, which also get the tag moiety. Unfortunately, MS-based imaging techniques have not reached the point of being quantitative so far, and are often use in combination with QWBA which remains the technique of choice for absolute quantification from tissue sections.^{96, 194}

Studies to address direct quantification by mass spectrometry-based imaging are currently on-going and are discussed on the 5th Chapter of this manuscript.

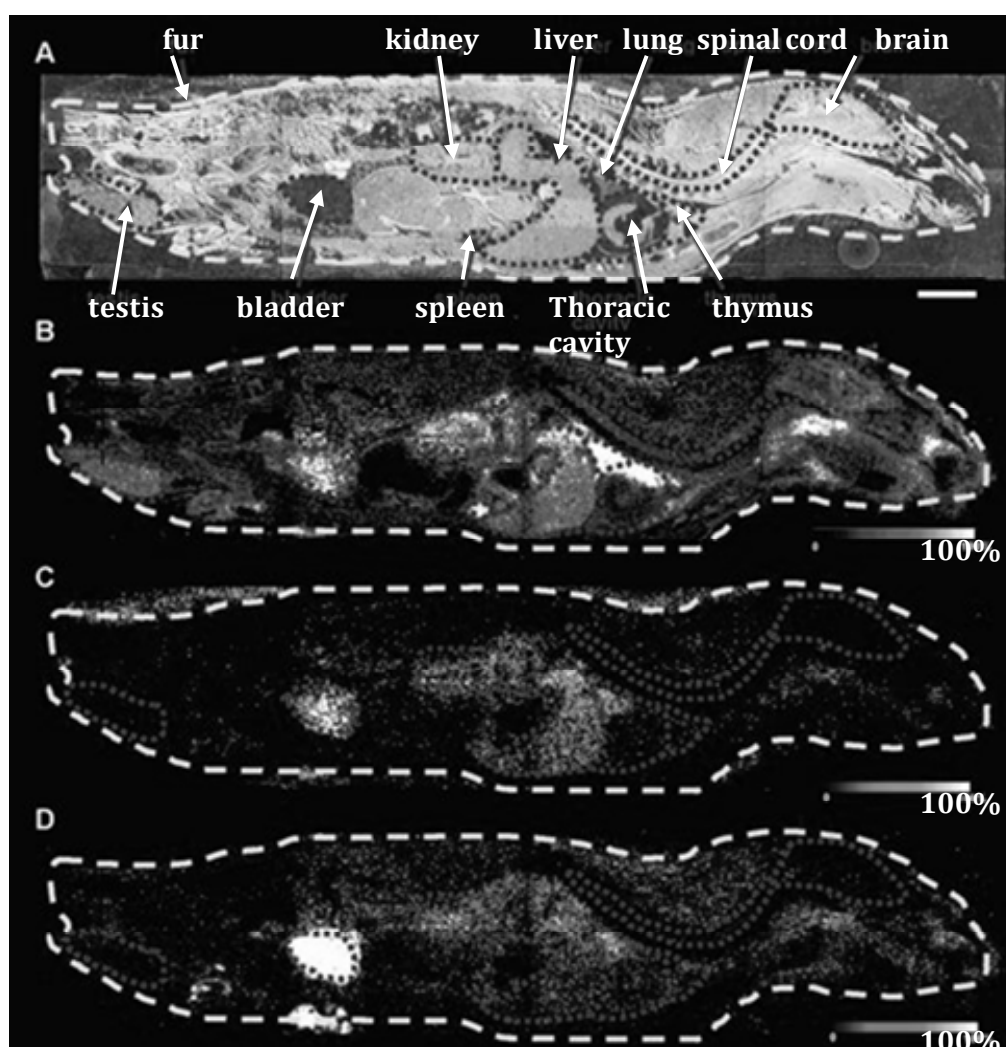


Figure I- 11. Detection of drug and metabolite distribution at 6 h postdose in a whole rat sagittal tissue section by a single IMS analysis. Optical image of a 6 h post OLZ dosed rat tissue section across four gold MALDI target plates (A). Organs outlined with dotted lines. MS/MS ion image of OLZ (m/z 256) (B). MS/MS ion image of N-desmethyl metabolite (m/z 256) (C). MS/MS ion image of 2-hydroxymethyl metabolite (m/z 272) (D). Bar, 1 cm. From ref.¹⁶⁷

1.1.8.5. Multimodal MSI

Multimodal approaches are often combined to get more information about the composition of the surface of a thin tissue section. To date, the most commonly used techniques for analyzing tissue surfaces are matrix-assisted laser desorption/ionization (MALDI) and secondary ion mass spectrometry (SIMS). The combination of these two techniques allows for collecting information about a wide range of molecular weight compounds, from elements to small molecules (SIMS) to higher molecular weight compounds such as phospholipids/peptides/proteins (MALDI).^{186, 195-196} In addition, SIMS allows for submicron imaging,¹⁹⁷ but, its sensitivity rapidly decreases with increasing mass such that these high-resolution images are typically obtained from ions having an m/z under 500.³

Recent studies have also demonstrated the suitability of direct surface analysis based on MSI in combination with other imaging modalities, such as laser capture microdissected cells,¹⁹⁸ immunochemistry,¹⁹⁹ magnetic resonance imaging,²⁰⁰ optical microscopy²⁰¹⁻²⁰³ or QWBA to accurately perform absolute quantification, which is not the case for MSI so far, as it will be discussed later in this manuscript.^{96, 194}

1.1.8.6. Limitations of MSI for the validation of cancer biomarkers

The analysis of proteins by MSI is continuously evolving as high-throughput in biomedical research. However, despite the advantages provided by MSI over the other imaging modalities, the identification of biomarkers is not straightforward and requires the use of sophisticated bioinformatics tools.²⁰⁴⁻²⁰⁵ A deep knowledge of statistical analysis tools is required to accurately interpret the huge volume of data generated. This allows the discrimination of peaks of high importance from cancerous to noncancerous tissues. The identification of these peaks also has to be carefully interpreted for in order to find potential biomarkers, reflected by differentially expressed proteins. By definition, statistics require a large set of data to validate biomarkers. However, in the most of the study performed, very few samples are available and statistical analysis suffers from the lack of relevant “real-cases” that should be a representation of the population studied.¹¹⁹ Fortunately, histologically compatible protocols have been developed for MSI, such as FFPE and formalin-fixed tissues^{139, 141-142} that are routinely used by pathologists.

Thus, hospital tissue banks represent a valuable archive of diseased tissues that could be further analyzed by molecular imaging based on mass spectrometry to provide larger set of samples to validate biomarkers (see Chapter 1, Part 2).

1.1.8.7. Development of ambient mass spectrometry for intraoperative molecular diagnosis - Real-time analysis of tumors and bringing MS in the surgery room

Although MALDI imaging has been the most widespread technique so far, emerging ionization techniques are being used particularly employing ambient ionization (see Chapter 2).²⁰⁶ New developments are on-going to provide rapid information of the

molecular composition of biopsy, and also to bring MS into surgery room,²⁰⁷ to assist surgeons during operation in real-time. This allows for providing rapid identification of biological tissues, of utmost importance for diagnosis and surgical treatment of various forms of cancer, and provides a new perspective for direct *in situ*, real-time identification of biological tissues based on mass spectrometry, so far limited to biopsy or postmortem analysis.

First, as a proof of concept, desorption electrospray ionization (DESI) mass spectrometry has been used on tumor or brain biopsy in combination with multivariate statistical analysis and machine learning to classify rapidly cancerous tissue (e.g. gliomas) and thus to provide surgeons, pathologists, and oncologists real-time information to guide surgical resections that can improve management of patients with malignant brain tumors.¹⁵¹ DESI has also been employed to directly analyze intact skin (i.e. by sampling at the tip of fingers) and has shown the potential of ambient mass spectrometry to be used at the time of the surgery.²⁰⁸

As an alternative to imaging methods, the so-called rapid evaporative ionization MS (REIMS) has been developed and appears to be promising in the field of intraoperative molecular diagnosis since it allows for *in situ*, real-time identification of biological tissues by both ultraviolet and infrared laser desorption ionization mass spectrometry and aims at minimizing the invasiveness of the operation.^{152, 209-210} REIMS combines laser desorption ionization mass spectrometry with the surgical CO₂ laser²¹¹ and allows the intra-surgical identification of tissues, and therefore, the localization of malignant tissues on the surgical area. REIMS is based on the electrosurgical evaporation of the tissues, where the ions formed during dissection are subsequently analyzed (aspiration through a Venturi air jet pump system (Figure I- 12a)).²¹⁰ In brain surgery, apart from laser surgery, another group of surgical methods employs mechanical disintegration of the tissues by ultrasound and vacuum suction to remove resulting tissue debris. This technique has also been developed for the real time analysis of brain tissue by direct combination with sonic spray mass spectrometry (Figure I- 13).²¹²

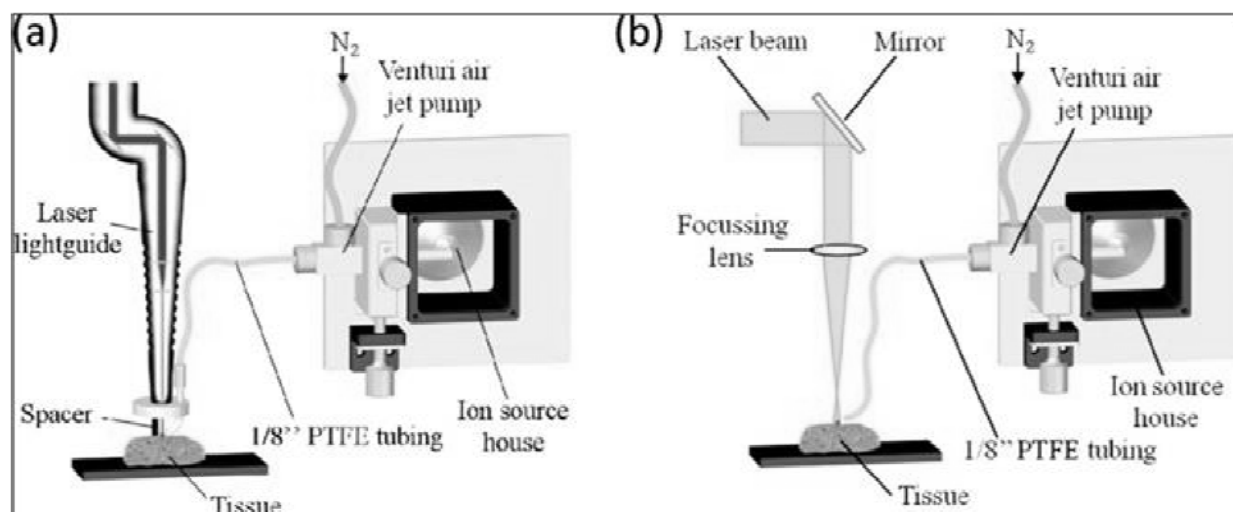


Figure I- 12. Experimental setups for the LDI-MS investigation of native biological tissues. The partially charged aerosol formed upon laser ablation is transferred to the atmospheric inlet of the mass spectrometer through a PTFE tubing by the suction force of a Venturi air jet pump. (a) Experimental setup for measurements with a surgical CO₂ laser. (b) Experimental setup for measurements with a Nd:YAG laser. From ref.²¹⁰

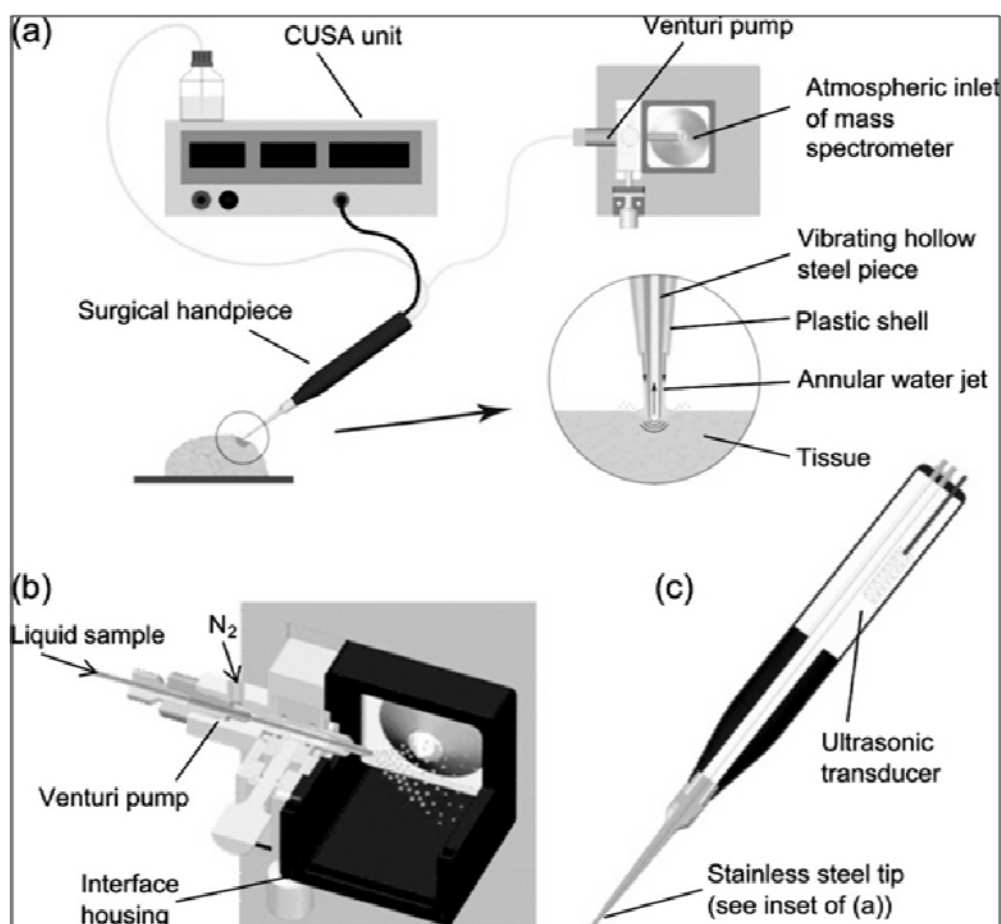


Figure I- 13. Real time analysis of brain tissue by direct combination of ultrasonic surgical aspiration and sonic spray mass spectrometry. (a) Experimental setup for the coupling of an ultrasonic surgical handpiece to a mass spectrometer. Inset shows tip of surgical handpiece. Arrows indicate water flow (down) and evacuation of tissue debris (up). (b) Detailed scheme of V-EASI-type setup mounted in front of mass spectrometer inlet. (c) The scheme represents the surgical handpiece. From ref.²¹²

1.1.9. Conclusion and perspectives

Medical, biomedical research is rich of a wide variety of imaging modalities that help to better understand and characterize diseases and there is no doubt that medical imaging will continue to expand.

The unique field of specific radiotracer development is a vast research area itself, which is undoubtedly going to be still further developed in the future. Nanotechnologies is an emerging tool in biomedical imaging for diagnostics and therapeutics will most probably play a major role in providing new diagnostic and therapeutic techniques.²¹³ This huge field of research has been deliberately omitted in this general introduction, which first aim is to expose a general overview of the main imaging techniques used in medical research.

Multimodal imaging allows for the combination of anatomical, functional, metabolic and molecular information and provides precious understandings into disease mechanisms, but also into mechanisms of drug action. Biomedical imaging offers a wide range of applications, from screening, diagnosing humans to pre-clinical and clinical research and finally to drug discovery and development. The use of biomedical imaging to measure and evaluate normal biological processes, pathogenic processes or pharmaceutical responses to a therapeutic treatment attains its limitation by the need of specific radiopharmaceuticals, which implies that prior knowledge about the sample composition is required.

This is where mass spectrometry imaging has a major role to play. By providing chemical specificity via the analyte's mass and localization by the position-correlated detection, without any prior knowledge of the imaging target, mass spectrometry imaging allows not only for a targeted analysis like the others techniques but also for the discovery of unknown species. Distinguishing a parent drug administered to its metabolites and accurately determining the spatial localization of both, is another example of benefit provided by MS imaging approaches.

If MSI was mainly applied so far to postmortem analysis or biopsies, recent developments allowed real-time analysis of tumors by bringing the MS technology in surgery room.

The keys to success of mass spectrometry imaging in the field of biomedical imaging rely on advanced instrumental developments that have pushed the limits in terms of ultra-high spatial resolution, high throughput, and high sensitivity to provide a better understanding of the underlying biology of health and diseases. These advanced developments in mass spectrometry imaging are reviewed in the second part of this general introduction.

1.1.10. Appendix: Summary of the techniques employed for biomedical imaging and their main characteristics

The following table is adapted and completed from references [1, 26, 57]

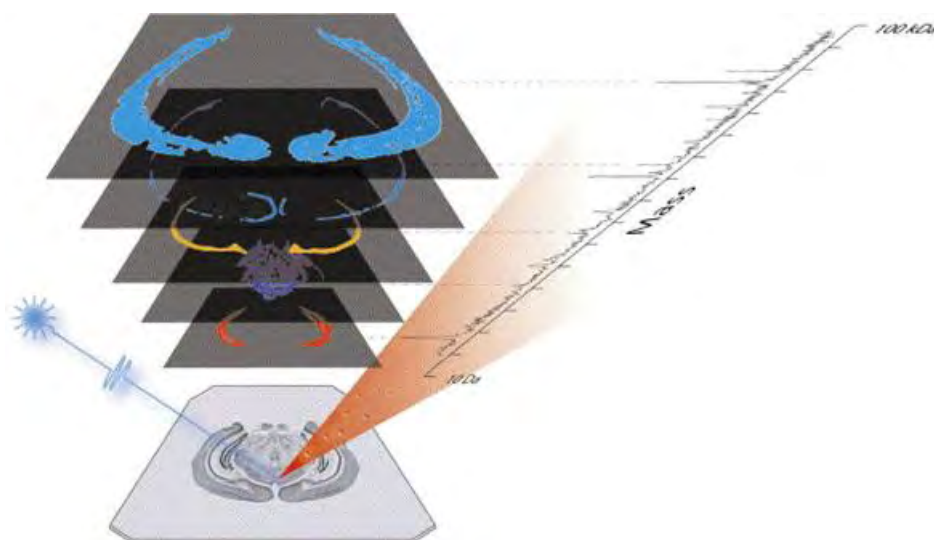
Techniques (+ synonyms)	Type of image	Basics principle	Spatial Rs	Depth	Sensitivity (M)	Endogenous contrast	Exogenous contrast agents used	Amount of probe
Magnetic resonance imaging (MRI) (= Magnetic Resonance Tomography MRT; Nuclear Magnetic Resonance (NMR) Imaging)	Nuclear (3-D)	Registration of change in electromagnetic properties of hydrogen or carbon atoms within a high strength static magnetic field	25-100 μ m	no limit	10^{-3} - 10^{-5}	yes	Gadolinium chelates; iron nanoparticles; small molecules containing ^{19}F or ^{17}O ; small diamagnetic or paramagnetic compounds containing exchangeable protons	μ g-mg
Functional MRI (fMRI)	Nuclear		1 - 5 mm					
Ultrasonography	(3-D)	Differential transmission and reflection of ultrasound waves by tissues	50-500 μ m	mm, cm	-	yes	Mircobubbles	μ g-mg
Contrast-enhanced ultrasound								
Echocardiography		Typical frequency ranges from 3-5 MHz up to 30-50 MHz						
Optical imaging	Molecular	Detection of light produced by biological or chemical moieties Can use one of the following techniques: absorption, scattering, single photon or 2 photon imaging, fluorescence, bioluminescence	20 mm	1-2 cm	-	yes	Substances which absorb or fluoresce in the visible or near-infra red region of the electromagnetic spectrum	Very low concentrations
Bioluminescence tomography (BLT)	Molecular	Detection of photons resulting from enzymatic reaction of luciferase/luciferin in living organisms	1-2 mm	1-2 cm	10^{-15} - 10^{-17}	yes		
Fluorescence tomography (FLT) (in small animals)	Molecular	Detection of visible and infrared light resulting from excitation of fluorophores with light of certain wavelength	1-3 mm	<1 cm	10^{-9} - 10^{-12}	yes (NADPH, green fluorescent protein)	Molecules tagged with fluorescent moieties	μ g-mg
Fluorescence resonance energy transfer or Förster resonance energy transfer (FRET)	Molecular	Uses the energy transferred between two fluorophores						
Fluorescence microscopy	Molecular	Detection of visible and infrared light resulting from excitation of fluorophores with light of certain wavelength	Angstrom = molecule	-	-			

Techniques (+ synonyms)	Type of image	Basics principle	Spatial Rs	Depth	Sensiti- vity (M)	Endogenous contrast	Exogenous contrast agents used	Amount of probe
X-Ray imaging (= X-radiography)	Nuclear (planar)	Differential absorption of X-rays by tissue components	50-200 μm	no limit	-	yes	Sometimes = molecules rich in high-Z atoms such as iodine, barium, xenon or thallium Might be platinum containing anti- cancer drug	-
X-ray computed tomography (CT)	Nuclear (3-D)	Measure accurately the electron density						
Fluoroscopy						yes		
Dual-energy X-ray absorptiometry (DEXA)		Same as X-ray				yes		
Planar nuclear imaging = Gamma-camera = Scintigraphy	Nuclear (planar)	Radioactive decay with emission of gamma ray				no	Detection of γ- photons emitted from radiotracers with γ-emitting	
Single-photon emission computed tomography (SPECT)	Nuclear (3-D)	Radioactive decay with emission of gamma ray	0.1-2 mm	no limit	10^{-10} - 10^{-11}	no	isotopes: ^{99m}Tc , ^{133}Xe , ^{67}Ga , ^{111}In , ^{123}I , ^{131}I , ^{51}Cr , ^{169}Yb , ^{81m}Kr	ng
Positron emission tomography (PET)	Nuclear (3-D)	Detection of pairs of γ-rays emitted after the annihilation of positron	0.2-2 mm	no limit	10^{-11} - 10^{-12}	no	The positrons are originated from β+ decay of positron-emitting radiotracer: ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{64}Cu , ^{124}I	ng
Macro- autoradiography Whole body autoradiography (WBA) or quantitative whole body	Nuclear	Measurement of the overall radioactivity (fluorescence)	< 50 μm					
Microautoradio- graphy (MARG)	Nuclear		< 10 μm					
Mass spectrometry imaging (MSI)	Molecular (2-D or 3-D)	Based on the measurement of mass-to- charge ratio of ionized molecules experiencing electric fields when entering the mass analyzer	< 1 μm (depends strongly on the ionization source)		Down to 10^{-12} - 10^{-15}	-	-	-

CHAPTER 1.

Imaging in biomedical research and the role of MSI

Part 2 – Advanced developments in MSI



1.2.1. Introduction

Since the introduction of the concept in the late 1990s by Caprioli's group²¹⁴⁻²¹⁵ the interest for molecular mass spectrometric imaging (MSI) has continuously grown in biomedical and clinical research.^{170, 216-217} The investigations focus on various areas such as proteomics,^{134, 215, 218-220} lipidomics,²²⁰⁻²²² metabolomics,²²³⁻²²⁵ pharmaceutical research and drug discovery.^{91, 143, 192-193, 226}

The unique goal of these approaches is to provide a better insight and characterization of the biological environment surrounding us, to explain diseases or metabolic disorders. MSI is also used to support drug discovery and development in understanding pharmaceutical activities, toxicological effects and distribution to determine sites of action of a given drug.^{91,193}

Currently, techniques based on liquid or gas chromatography (LC or GC) with mass spectrometric detection are used to identify and quantify analytes. However, these analyses are only possible from tissue homogenates and are limited to the scale of an organ. This limitation leads to the loss of the spatial integrity of the samples, and thus the spatial information. In addition, the determination of drugs from plasma or whole-blood samples does not accurately reflect the concentration in tissue. To achieve cell analysis, the complex tissue samples have to be microdissected, which relies on time consuming sample preparation.¹¹¹ One of the strengths of mass spectrometry imaging-based approaches is the possibility to analyze directly tissue sections, which thus does not require tissue homogenization.

As specified previously in Chapter 1, Part 1, and contrary to other imaging techniques such as whole body autoradiography (WBA), mass spectrometry-based imaging techniques are label-free and rely on the desorption of molecules present on the surface of a complex sample. The specificity of mass spectrometry allows for accurate determination of the localization of parent compounds and metabolites in a single experiment. Co-localization of drug distribution and endogenous analytes identified as biomarkers traducing disease progression, therapeutic or toxicological effect.¹⁹³

The success of mass spectrometry imaging in biomedical imaging and drug development and discovery rely on extensive efforts focused on instrumental developments to push the limits of ultra-high spatial resolution, throughput, and sensitivity. As a consequence, the mass spectrometry imaging enables to:

- i) obtain molecular information down to cellular level, that is employed in cancer research to distinguish healthy from cancer cells for instance;
- ii) generate, acquire and process data in a fast fashion;
- iii) detect both targeted and untargeted compounds even at low abundances.

Because of the large number of published papers in the MS imaging area from 1997 (more than 3770 articles can be found on the web, as shown Figure I- 14, this manuscript does not attempted to make this part all-inclusive. This objective of this part is to highlight a number

of key advanced developments that have contributed to make MSI a competitive and powerful approach, particularly in drug discovery and development.

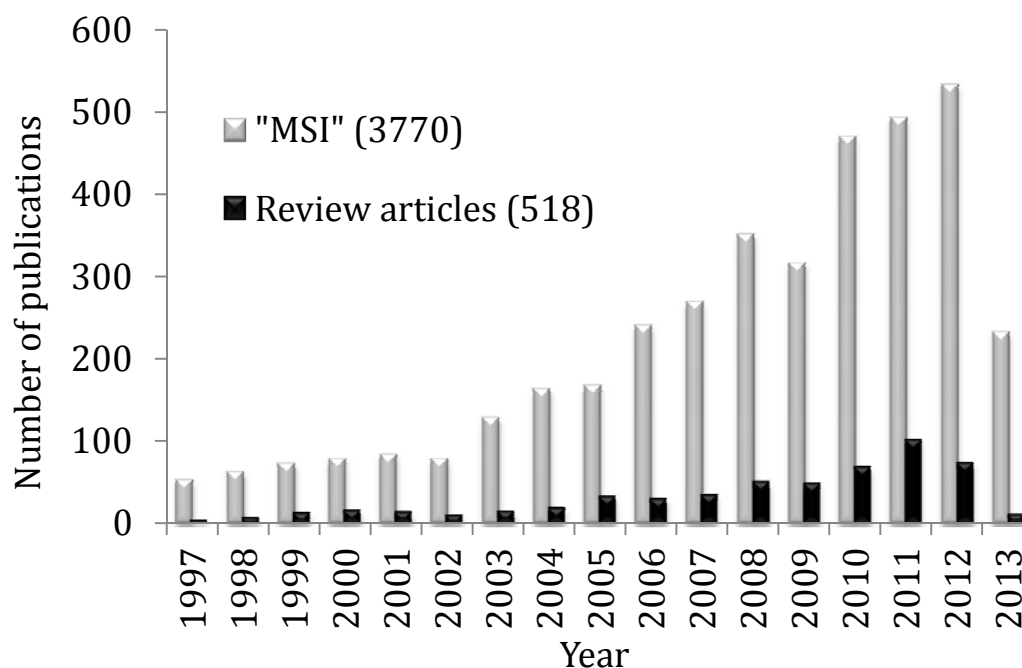
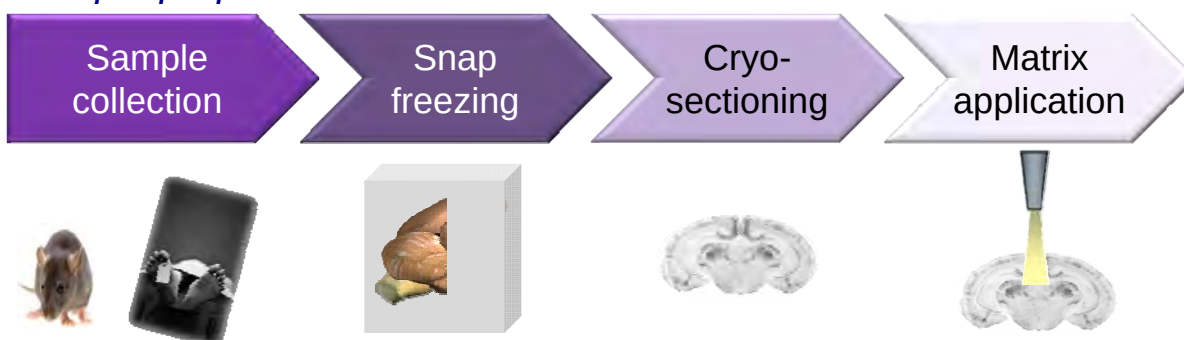


Figure I- 14. Yearly histogram of publications dealing with “mass spectrometry imaging” after the introduction of the concept by Caprioli in 1997. The information was collected by searching SciFinder on the 09.05.2013 for the entry “mass spectrometry imaging”. In blue: total number of articles containing the concept “mass spectrometry imaging”. In red: number of review articles only.

The general analytical workflow common to all MS-based molecular imaging techniques is presented Figure I- 15 and consists basically in four steps, i.e. sample preparation, sampling of analytes from a surface and ionization, mass analysis and data processing. The sample preparation includes the sample collection and handling. The application of a matrix is optional (depending on the ionization technique used) and is by far the most critical step to optimize, in order to generate relevant MS images. After desorption/ionization of the biological material, full MS or MS/MS spectra are acquired. Simultaneously, the relative abundance and spatial location of molecular species are recorded. A 2-D map is then reconstituted, reflecting the molecular distribution on the sample surface. Signals can be acquired either for tissue profiling (i.e. from selected spot(s)) or for imaging selected areas or entire tissue. If matrix-assisted laser desorption/ionization (MALDI) is by far the most widely used ionization techniques, recent developments have pushed forward the use of ambient ionization techniques. These techniques are mostly matrix-free techniques, and aims at considerably reducing and simplifying the sample preparation and handling. Each stage of the process has to be carefully designed to maintain the spatial integrity of the sample (i.e. sectioning, matrix application) and to generate MS images “fit-for-purpose” more or less resolute in function of the objective of the study.

Sample preparation



Mass spectrometry analysis

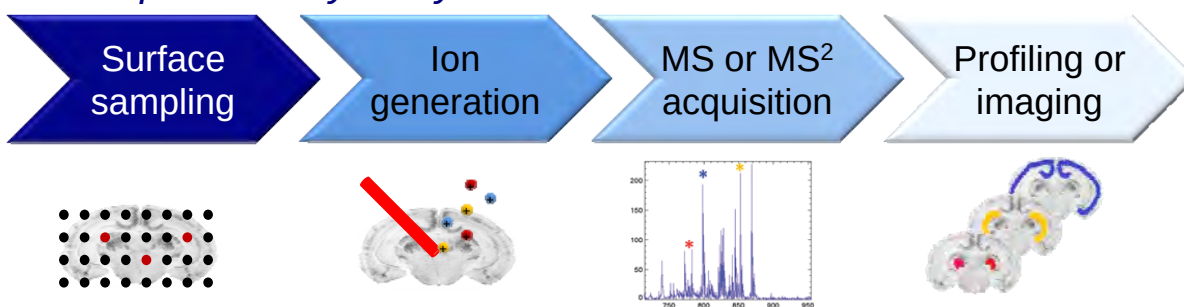


Figure I- 15. General analytical workflow common to all MS-based molecular imaging techniques. The upper part shows the sample preparation workflow. The lower part presents different steps of the mass spectrometry analysis workflow.

1.2.2. Sample preparation in MSI: practical considerations

1.2.2.1. Sample collection and sample handling

The sample preparation is a critical step in the MS imaging workflow. The sample preparation has to be carefully optimized to reflect what is going *in situ* and “*small mistake can lead to big consequences*”.²²⁷ MSI can be achieved either on single cell, single organ, tissue biopsy or entire animal. In human, only tissue biopsies or postmortem investigations can be performed, for forensic toxicological investigations for instance. Alternative matrices such as hair samples can also be analyzed by MSI approaches.²²⁸⁻²³¹

The way to collect and handle the tissue of interest is particularly critical, especially for the analysis of metabolites distribution because postmortem degradation can occur. To prevent rapid changes of tissue constituents due to autolysis and/or enzymatic reactions, enzyme activities can be inactivated by quick fixation methods. These techniques can be focused microwave irradiation or flash-freezing (e.g. in liquid nitrogen at -80°C) immediately after sacrifice/ablation. To maintain the flat shape of the sample, and depending of the nature of the sample and the feasibility, the sample can be placed in-between 2 aluminum foil prior flash-freezing.²³² However, the freezing process may lead to sample cracking and deterioration. Usually, samples are stored at -80°C prior to the analysis, for at least one year

with no significant degradation. The exposure of the sample to the light must be avoided as much as possible to prevent possible degradation of photosensitive samples.²³³

The sample is then sectioned and the thickness of the sections depends on the tissue and also the area of interest. Whole body sections of rodents are typically cut at 20-50 μm due to practical and critical handling. Single organs are cut at 5-20 μm , which matches the size of a mammalian cell (i.e. *ca.* 10-20 μm). As a result, the majority of the cells are expected opened to expose the intracellular content for the analysis.²³³⁻²³⁴ The main issue encountered with thick sections is due to the non-conductive property of the biological material. Thick material may interfere with the measurement process and hamper the inefficiency of the analyte ionization. This can be overcome by coating a thin layer of gold at the surface of the sample.²³⁵ In addition, improved peak intensities and signal-to-noise ratios for proteins were demonstrated by the direct analysis of thinner slices (i.e. < 10 μm).²³⁶ It's noteworthy that the sectioning of whole body small animals required specific instrumentation to cut the whole specimen, which has to be embedded in appropriate embedding medium (e.g. carboxymethylcellulose,²³⁷⁻²³⁸ gelatin,²³⁹ ice¹⁶⁷). The transfer to adequate support (usually using double-sided adhesive tape) is also critical. Smaller sections from selected organs can be directly thaw-mounted onto sample holder which can be regular glass slides, ITO (indium-tin oxide) coated glass slides, or stainless steel or gold-coated plates.

1.2.2.2. Washing protocols and additional treatment for proteomic analysis

Although the analysis of low molecular weight compounds usually do not require additional pre-treatment, a tissue clean-up step is required for proteomic analysis.^{138, 227} The detection of protein/peptides can be hampered by the presence of abundant tissue components such as phospholipids or salts from biological tissues. Tissue-washing procedures use organic solvent such as chloroform, xylene, hexane,²⁴⁰ ethanol/water²⁴¹ or isopropanol.²⁴²⁻²⁴³

If these washing protocols considerably increase proteins and peptides signals, they cannot be applied to small drugs compounds analysis. Such compounds are usually soluble in the solvents used for washing the tissue section and then they would also be washed out from the tissue, and then delocalized. Some attempts have been performed to image by MALDI-MSI the distribution of two pharmaceuticals (i.e. cimetidine and imipramine) and one new protease inhibitor after washing tissue sections using a pH-controlled buffer solution. This strategy consists in using pH where analytes have low solubility. Minor to no delocalization of the analytes were observed, along with a sensitivity enhancement by a factor of up to 10.²⁴⁴ Finally, washing the tissue may improve the crystallization process of the matrix.^{234, 245-246}

On-tissue tryptic digestions protocols have been developed either from frozen or FFPE tissues^{133-134, 139, 141-142, 171, 247-248} to improve bottom-up identification of biomarkers in tissues. The main limitation of these approaches is they typically allow identification of the most abundant proteins. To overcome this issue, strategy combining on-tissue trypsin

digestion followed by micro-extraction using a liquid micro-junction interface was developed to obtain identification of proteins of various abundances while keeping their localization within the section.²⁴⁹

Recently, a mass spectrometry compatible heat stabilization technique has been developed for use in tissue fixation (Stabilizor™, Denator AB, Sweden) where the tissue is rapidly heated at 95°C. This approach combines heat and pressure under vacuum, which inactivate enzyme activity. The results prove homogenous and reproducible procedure and prevent downstream sample degradation, commonly encountered with other techniques (e.g. snap-freezing). This technology was applied for improving proteomics by greatly reducing the complexity and dynamic range of the proteome²⁵⁰ and for the localization of adenine nucleotides mouse brains.²⁵¹ So far, it has not been evaluated for the analyte of exogenous compounds.

1.2.2.3. Choice of the matrix for matrix-assisted ionization techniques and application techniques

Matrix-assisted laser desorption/ionization (MALDI) is the most widely used technique for MS imaging of small and large molecules. The choice and application of a matrix to promote the analyte's ionization is a crucial step for all matrix-assisted techniques. It may affect the sensitivity, the spatial resolution as well as the analyte response factor. The choice of the organic solvent (acetonitrile or methanol) used for dissolving the matrix also plays an important role for the analyte extraction from the tissue. The way the solvent evaporates also impacts the size of the matrix crystals formed: fast evaporation leads to smaller crystals.²⁵² In addition, some cautions should be taken during application of matrix solution application: on one hand, the spray conditions (i.e. solvent composition, flow, speed, distance between the sprayer tip and the sample) have to be optimized to generate a spray which is wet enough to extract the analytes from the tissue section and on the other hand, the spray must not be too wet to avoid their delocalization within the different region of the tissue.

The matrix must be chosen in function of the mass range of the analytes as well as the chemical properties of the analyte. Typically, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) is preferred for the analysis of large molecules such as proteins,²⁵³ while α -cyano-4-hydroxycinnamic acid (CHCA) is well appropriate for peptides²⁵⁴ and low molecular weight compounds (i.e. pharmaceuticals, drugs of abuse, metabolites).^{143, 191} 2,5-dihydroxybenzoic acid (DHB) is also used for imaging small molecules¹⁶⁷ and is particularly suited for lipids. Finally, 9-aminoacridine (9-AA) is the matrix of choice for small compounds analysis in the negative ion mode.²⁵⁵⁻²⁵⁶

Although the use of these matrices is well established for specific application, matrix interferences remain the main issue in the low m/z range, which is critical for small molecules analysis and the quest for the perfect matrix is still in progress.²⁵⁷⁻²⁶⁰ Recently, 4-

chloro- α -cyano-hydroxycinnamic acid (Cl-CCA) was demonstrated suitable for increasing sensitivity and considerably enhancing peptide recovery in proteomic analyses because of a much more uniform response to peptides of different basicity^{248, 261-262} as well as to improve the sensitivity for some pharmaceuticals compounds.²⁵⁷

Some examples of developments of alternative matrices are given here: proton sponge matrices (1,8-bis(dimethyl-amino)naphthalene; DMAN) generate spectra completely free from matrix peaks;²⁶³⁻²⁶⁴ ionic (liquid or solid) matrices²⁶⁵⁻²⁷⁰ lead to higher peptide signal intensity and sensitivity, reproducibility and higher resistance to laser ablation;²³⁴ binary mixtures.²⁷¹ Nanoparticle (NP)-assisted laser desorption/ionization (nano-PALDI)-based IMS techniques are also employed, in which NPs are used as the ionization-enhancing reagent and the organic matrix crystallization process is eliminated, and allow reduced chemical background.^{174, 272-273} These nanoparticles can be iron oxide,²⁷⁴ silver²⁷⁵ or gold²⁷⁶ and have found applications for lipid-related compounds (i.e. phospholipids, fatty acids, glycosphingolipids) and carbohydrates. Also, solvent free-methods (i.e. dry matrix application) are alternate approaches that minimize the potential delocalization of lipids and pharmaceuticals in tissues.²⁷⁷⁻²⁸⁰

Because the typical matrix is usually a small organic compound co-crystallizing with the analytes extracted from the sample, different parameters have to be considered. This is to keep intact the spatial integrity of the sample and avoid the delocalization of the analytes within the tissue. These parameters are: the size of crystals (which impacts the lateral resolution), the homogenous coverage of the sample, the extraction efficiency of analytes from the tissue and the accessibility of matrix solutions to the lower layers of the tissue, the process of recrystallization of matrix together with the extracted analytes during the solvent evaporation.²⁸¹ To improve consistency from one analysis to another, the amount of matrix deposited must be carefully controlled and be reproducible, explaining the development of automated devices.

Table I- 7 reports some example of spatial resolution achieved with different MALDI matrix deposition methods, including manual and automated (spotting and spraying) methods.

Spotting techniques create larger spot size than spraying techniques (i.e. worse resolution), but enhanced the extraction efficiency of the molecules.²³³ Spotting is well suitable for tissue profiling, where imaging the whole surface of the sample is not mandatory. The spatial resolution is in that case limited by the size of the matrix spots, larger than the size of the ion beam. Spraying techniques produces smaller matrix crystals, which is compatible with high resolution imaging to image small features, and must be optimized to produce a homogenous matrix layer and avoid analyte diffusion. The best resolution is achieved with the sublimation method, which allows for imaging down to 10 μm -resolution with MALDI.²⁸²⁻²⁸³

Unfortunately, no universal technique exists so far and the sample preparation has to be optimized in function of the objective of the analysis.

Table I- 7. Spatial resolution achieved with different (MALDI) matrix deposition methods.
Adapted and completed from ref.^{193, 234, 281}

Type of spraying	Method	Principle/interest	Spatial resolution	Applications
Manual spotting	Micropipette	Manual deposition of μL droplets	> 1-2 mm	On-tissue digestion ^{137, 240}
Manual spraying	Thin layer chromatography (TLC) sprayer		< 1 mm	
	Pneumatic sprayer / Painter airbrush		< 1 mm	Proteins ²⁸⁴ Drugs ²⁸⁵
Automated spotting	Heated droplet interface	The liquid stream in the transfer tube is heated to a temperature sufficient to cause partial evaporation of the carrier solvent from the hanging droplets	0.8 mm	Peptides and proteins ²⁸⁶⁻²⁸⁷
	Robotic spotter	Mechanical spotting / injection as dot-matrix	~130-150 μm	Proteins ²³³
		<i>Commercial devices:</i> MALDI Sun Collect Spotter (SunChrom GmbH, Germany)	250 μm	On-tissue digestion ¹³⁷
	Piezoelectric-based inkjet printer ²⁸⁸	Deposition of 50-100 pL droplets (spot size of 100-200 μm) by micro-dispensing of fluid, preferably avoiding the contact with the tissue to avoid cross-contamination Clogging possible <i>Commercial devices:</i> ChIP-1000 (Shimadzu Co., Kyoto, Japan) Desktop inkjet printer ²⁸⁹	< 150-200 μm	On-tissue protease digestion ^{133, 290-295}

Table I- 7. Continued.

Type of spraying	Method	Principle/interest	Spatial resolution	Applications
Automated spraying	Heated capillary nebulizer + nebulization	Rapid on-line desolvation of effluent from flow rates of 200 nL/min to 50 nL/min		Peptides ²⁹⁶
	Vibration vaporization	ImagePrep (Bruker Daltonik GmbH, Bremen, Germany)		
	Acoustic deposition	Deposition of 100 pL droplets (spot size of 100-200 μm) using acoustic energy (sound wave) No capillary or nozzle for fluid ejection, so no issues with clogging of these orifices <i>Commercial devices:</i> RapidSpotter (Picoliter Inc., Sunnyvale, CA) Portrait 630 (Labcyte Inc., Sunnyvale, CA)	200 μm	Proteins ²⁴⁵ Drugs and metabolites ¹⁶⁸
	Electrospray deposition	Consists in consisted of a syringe pump, a fused-silica capillary with the electrospray emitter mounted on an XZ-translation stage Clogging of the nozzle possible	200-300 μm	Peptides ²⁹⁷ Polymers ²⁹⁸
	Nebulized spray coating	Pneumatic vaporization by pressed air, assisted by heat <i>Commercial devices:</i> HTX-TM sprayer (HTX Technologies, Chapel Hill, North Carolina, US)) (old: LEAP-TM sprayer) MALDI Sun Collect Spotter -sprayer (SunChrom GmbH, Germany)	~10 μm	Lipids ²⁹⁹ ; drugs ³⁰⁰ ; proteins ³⁰¹
	Sublimation	Sublimation is a solvent-free matrix deposition where the matrix (transformed from solid to gas state) is slowly applied by after application of heat (from 25 to 120°C in 10 minutes) in a heating mantle kept at reduced pressure (e.g. 0.05 Torr) ²⁸² <i>Commercial devices:</i> Sublimation apparatus 8023 (Ace Glass, Vineland, NJ)	5-10 μm	Imaging lipids in optic nerve tissue ²⁸³ HIV protease inhibitor ³⁰² Imaging neutral cuticular lipids in insects and plants ³⁰³
	Sublimation/ Recrystallization		10-50 μm	Proteins from rodent brain ²⁴¹

1.2.3. Surface sampling and strategies developed for high spatial resolution and high throughput MS imaging: microprobe versus microscope approaches

In mass spectrometry imaging, it is possible to acquire the data in two ways: the **microprobe mode** and the lately introduced **microscope mode** (Figure I- 16). This part reviews the strengths and weaknesses of these two approaches and particularly highlights the developments that have been made to improve the two strategies, and particularly to improve sample throughput, spatial image resolution and analyte detection.³⁰⁴

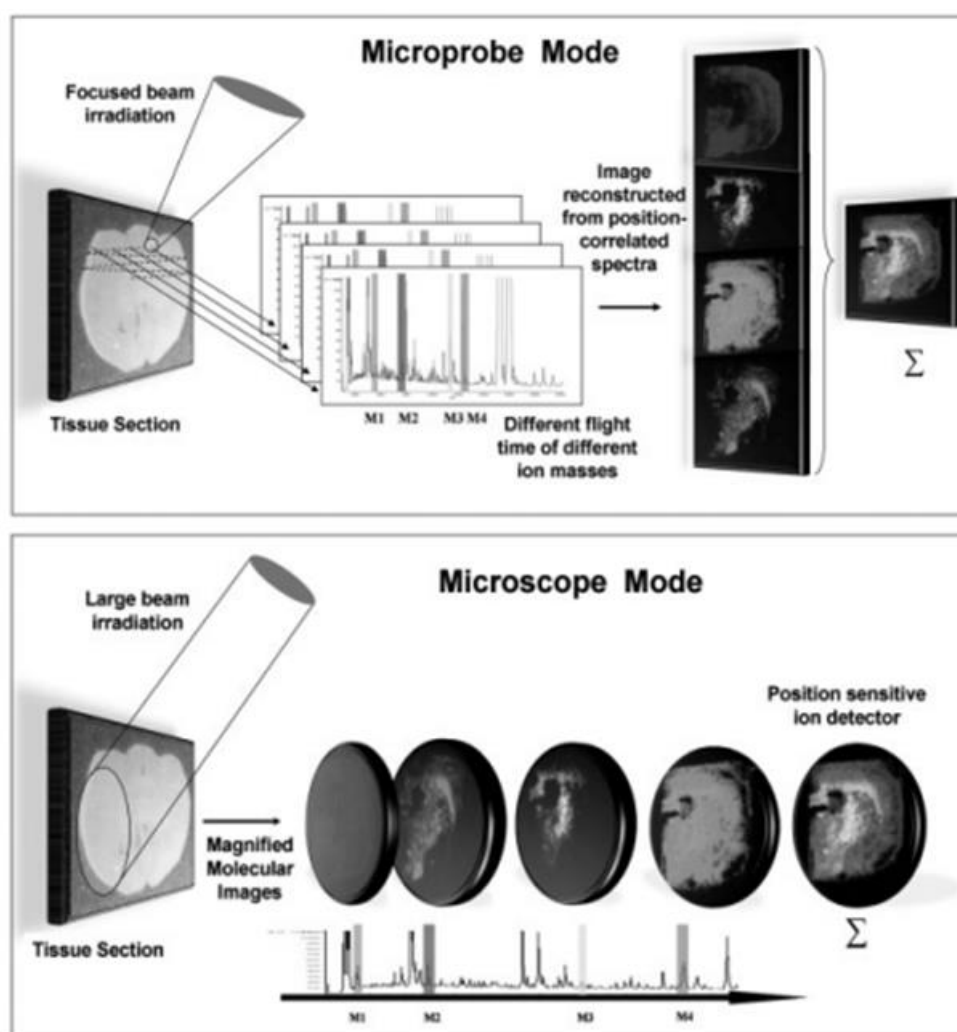


Figure I- 16. MALDI imaging in microprobe mode versus microscope mode. Microprobe is one MS per laser spot, whereas microscope is one set of images per laser spot. Hence, microscope enables high spatial resolution, lower than laser spot diameter. From ref.²¹⁸

1.2.3.1. Microprobe mode MS imaging

The design of experiment strongly influences the quality of the image generated, particularly the size of the pixel/voxel defined to produce the image. Most commonly, the microprobe mode is used to acquire MS image and one pixel corresponds to one MS experiment. There are 2 ways of acquiring images: the discrete mode and the rastering mode (Figure I- 17).

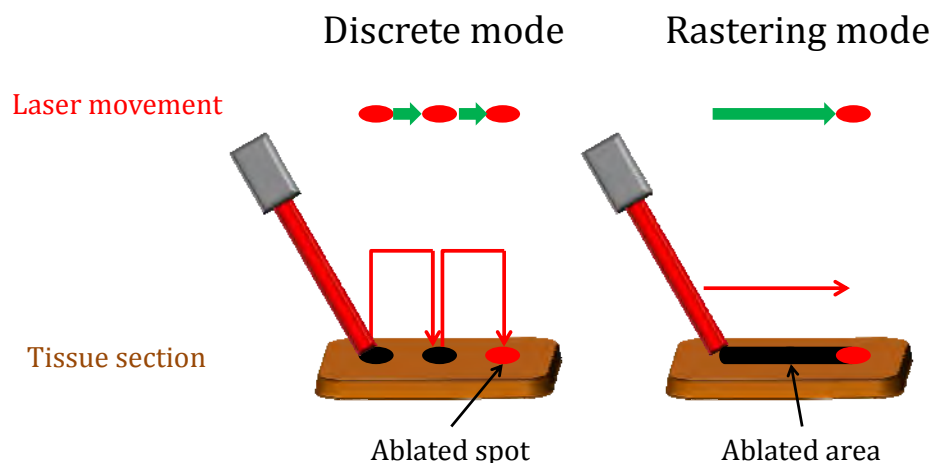


Figure I- 17. Schematic representation showing laser movement and sample ablation during spot-by-spot (discrete mode) and continuous raster (rastering mode) imaging. Adapted from ref.³⁰⁵⁻³⁰⁶

a) Discrete mode

The discrete mode is a spot-by-spot sampling technique and is well adapted for tissue profiling. It consists of ionizing sequentially spot areas at the surface of a tissue section. The ablated area is of the size of the pulsed desorption/ionization beam (i.e. laser beam in MALDI, primary ion beam in SIMS). This is the main factor of the spatial resolution achieved. The image is generated with pixels of which size correspond to the ionizing beam dimensions. To increase the spatial resolution of the image, developments have reduced the dimension of the surface area sampled by using highly focused primary ion or laser beams. If SIMS is particularly attractive for sub- μm resolution imaging,³⁰⁷⁻³⁰⁸ other techniques still need to be improved. Recent developments with a MALDI source operating at atmospheric pressure allows for returning pixel sizes down to the 1-10 μm range,³⁰⁹⁻³¹¹ and even down to 0.6 – 1.5 μm depending on the sample properties.³¹² This is possible by focusing of the laser beam, which consists in a pre-focusing part outside the vacuum and a final focusing part in the vacuum right above the sample manipulator.³¹²

However, it's noteworthy that decreasing the size of the area sampled affects the sensitivity and there is a compromise to make between resolution/sensitivity. The discrete mode has the main disadvantage of long measurement time, which depends to the mass analyzer duty cycle, the laser repetition rate and its fluence.

b) Rastering mode

To improve MS imaging performance, the continuous rastering approach has been introduced.³⁰⁵⁻³⁰⁶ The rastering mode consists in rastering continuously the surface of the sample with the laser (or ion beam). This technique improves acquisition time by a factor of 2.5 – 5 while simultaneously improving sensitivity. The rastering mode allows for high resolution images. Depending on the instrumentation design, the spatial resolution is a

function of the duty cycle of the MS experiment, the speed of the ionization beam or the sample stage, and the precision of the sample stage positioning (see Table I- 8). The performance of such approach was improved by the implementation of faster repetition rate laser (see next paragraph). As an example, together in combination with a 1 kHz laser, high sample stage velocity, the resolution of the image generated can be considerably improved using the selected reaction monitoring mode that enables fast acquisition of the ions generated (i.e. pixels of $50 \times 50 \mu\text{m}^2$ with a laser beam of $100 \mu\text{m}$ -width). See Chapter 3 Part 3 and Part 4 of this thesis manuscript for more detailed considerations. Figure I- 18 is a spectacular example of high quality MS imaging showing the distribution of lipids in a sagittal rat brain tissue section within 10 minutes, obtained from high speed MALDI-MSI experiments combining high sample stage velocity of 5 mm/s in continuous rastering mode and high repetition rate laser of 3 kHz.

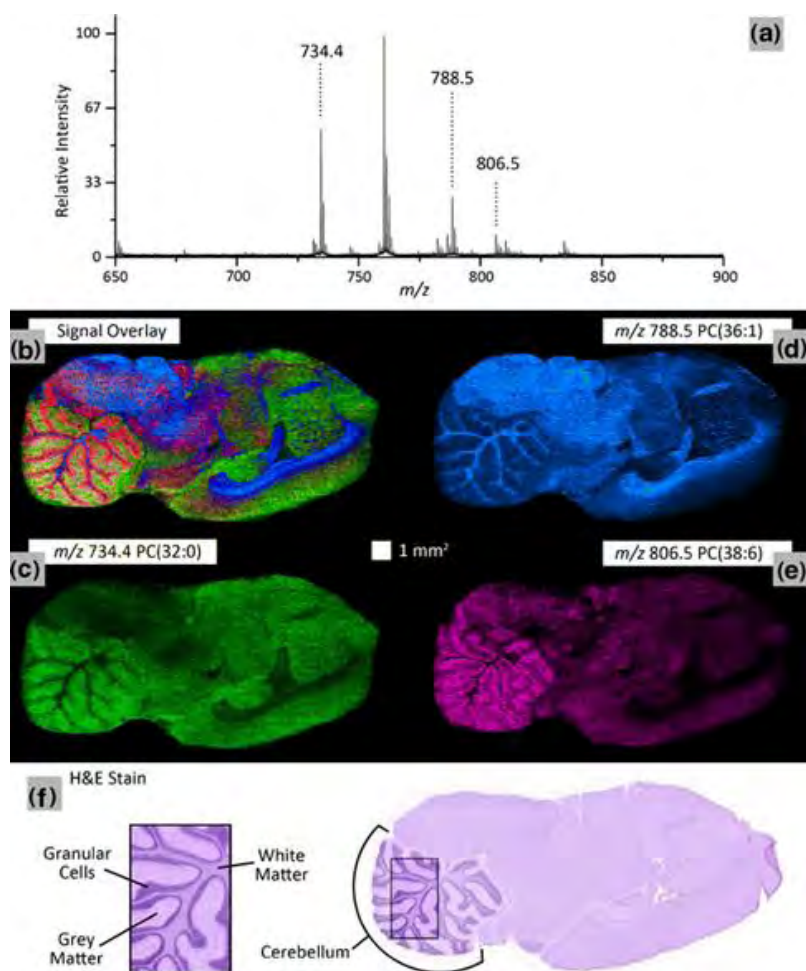


Figure I- 18. A $100 \mu\text{m}$ spatial resolution lipid ion image of a sagittal rat brain tissue section using typewriter continuous laser raster sampling acquired in 10 min. (a) Representative spectrum is shown. The ion image overlay (b) of signal from m/z 734.4 PC(32:0) (c), m/z 788.5 PC(36:1) (d), and m/z 806.5 PC(38:6) (e) highlights the differentiation of the spatial distributions for the selected ions. These results correlate to the H and E stained serial tissue section highlighting structural difference between grey matter, white matter and granular cells in the cerebellum (f). Important instrumental parameters: 3 kHz laser repetition rate, 5 mm/s sample stage velocity, and 60 laser shots/spectrum hardware average. From ref.¹¹⁸

c) Importance of the laser and development of high repetition rate lasers

Several design considerations have to be considered increasing the speed of measurements on a MALDI-MS (imaging) experiment, and the ion generation is one of them. Particularly, generating maximum ion signal intensity as fast as possible is of utmost importance. If the majority of the experiments have been performed so far with nitrogen lasers (N_2 , 337 nm), their operating frequency is usually from 20 to 60 Hz (Table I- 8). One of the major drawbacks of N_2 lasers is to have irregular beam profiles with many and varying hot spots.³¹³ Solid state lasers have then enabled users to take advantage of improved reliability, extended lifetimes and consistently good beam quality. Such lasers consist in a crystalline that is "doped" with ions that provide the required energy states. Neodymium is a common dopant in various solid-state laser crystals, including yttrium orthovanadate (Nd:YVO₄), yttrium lithium fluoride (Nd:YLF) and yttrium aluminium garnet (Nd:YAG). All these lasers can produce high powers in the infrared spectrum at 1064 nm, but they are usually frequency doubled, tripled or quadrupled, to produce UV beams of which wavelengths are absorbed by common UV-MALDI matrices (see Chapter 3, Part 1). In order to improve the sample throughput, higher repetition rate laser, i.e. up to 20 kHz (Table I- 8), have been implemented in the instrumental design of MALDI-MSI. This allows production of a continuous beam of ions for use in combination with an orthogonal TOF instrument configuration,³¹⁴ or with faster continuous scanning mode, such as the selected reaction monitoring (SRM) available on a QqQ_{LIT} mass analyzer.^{191, 230} The most of the studies report an increase in sensitivity due to beneficial effect on both the ion production and the rate of its formation.³¹⁵

If UV-lasers are the mostly used, infrared (IR) lasers are also employed, such as the Er:YAG ($\lambda=2.94\ \mu\text{m}$, pulse of 50-150 ns).³¹⁶ Although MALDI spectra obtained from UV and IR laser wavelengths are similar,³¹⁷ IR-MALDI requires higher laser pulse energy due to the lower MALDI matrix absorption, which results in a higher consumption of the sample.³¹⁸

The choice of the laser as well as the influence of its intensity on the spot size will be discussed in Chapter 3, Part 1. This will have a strong impact on the spatial resolution in MSI experiments. To illustrate, and as discussed previously, in microprobe imaging, the spatial resolution is increased with decreasing laser spot size. However, as the rate of the energy redistribution rapidly increases with smaller laser spot sizes, high laser fluence are required for MALDI to occur, which can cause extensive fragmentation.³¹⁹ Laser spots focused to 7-8 μm lead to a decrease in ion yields of 2 orders of magnitude compared to a 100 μm laser spot, since the area sampled is *ca.* 100 times smaller. In addition, high spatial resolution experiments are more sensitive to analytes migration during the sample preparation (i.e. matrix application) and dramatically increase the analysis time for whole tissue section analysis.³²⁰

Table I- 8. Lasers and features. λ =wavelength.

Laser	Freq.	Pulse	Energy	MS analyzer hyphenated	Matrix used	Applications
Nitrogen N ₂ 337 nm	20-60 Hz	3-4 ns	300 μ J/ pulse	TOF, ion trap	Cinnamic acid and derived matrices (CHCA, SA), DHB	³¹²
Nd:YAG Frequency-tripled 355 nm	1 kHz	< 500 ps		AP-MALDI-QqTOF ³¹⁴ MALDI-QqQ _{LIT} ^{191, 230}	Cinnamic acid and derived matrices (CHCA, SA), DHB	Peptide (signal for Angiotensin II x 80) ³¹⁴ Small drugs (pharmaceuticals, drugs of abuse)
Nd:YAG Frequency-tripled 355 nm	2 kHz			MALDI-TOF ³²¹		
Nd:YLF Frequency-doubled 524 nm	20 kHz	15 ns	100 μ J	MALDI-TOF	DHB	Peptides ³¹²
Nd:YLF Frequency-tripled 349 nm	20 kHz (operated at 3 KHz for tissue imaging)	15 ns	5 μ J	MALDI-TOF	DHB, CHCA	Peptides, Lipid in a sagittal rat brain tissue section ¹¹⁸
Nd:YLF Frequency-quadruple 262 nm	20 kHz	15 ns	-			
Nd:YVO4 Frequency-tripled 355 nm	20 kHz	1.5 ns	7 μ J	Q-TOF	CHCA, SA	Rat brain, pancreatic tumor tissue ^{260, 322}

* Although the Nd:YVO4 laser can be operated up to 20 kHz, it has been demonstrated that the laser yield the best results for MALDI-MS imaging experiments when operating at repetition rates 5-10 kHz.³²²

d) MALDI-MS imaging of features smaller than the size of the laser: the oversampling method

When the laser beam size is the limiting factor for image resolution MALDI microprobe MSI, this limitation can be overcome by oversampling.³²³ In the oversampling method, the raster increment of the sample stage movement is smaller than the laser beam dimensions. This

results in the complete ablation of the sample. Therefore, $60\ \mu\text{m} \times 100\ \mu\text{m}$ resolution could be generated with a laser beam of $100\ \mu\text{m}$ wide by $200\ \mu\text{m}$ high.³²⁴ The oversampling strategy can be implemented on a spot-to-spot approach (i.e. discrete mode),³²⁵⁻³²⁷ as well as in the rastering mode. In the latter, the resolution can be improved in-between 2 line scan as shown Figure I-19. In our instrumental setup (i.e. prototype version of the commercial version of the Flashquant, AB Sciex, which consist in a MALDI source implemented in front of a triple quadrupole mass analyzer, see Chapter 3, Part 2), the limitation is the minimum step of the motor which is of $30\ \mu\text{m}$. To illustrate, an example of application in plant biology is given in the 3rd Chapter of this thesis manuscript (Part 3).

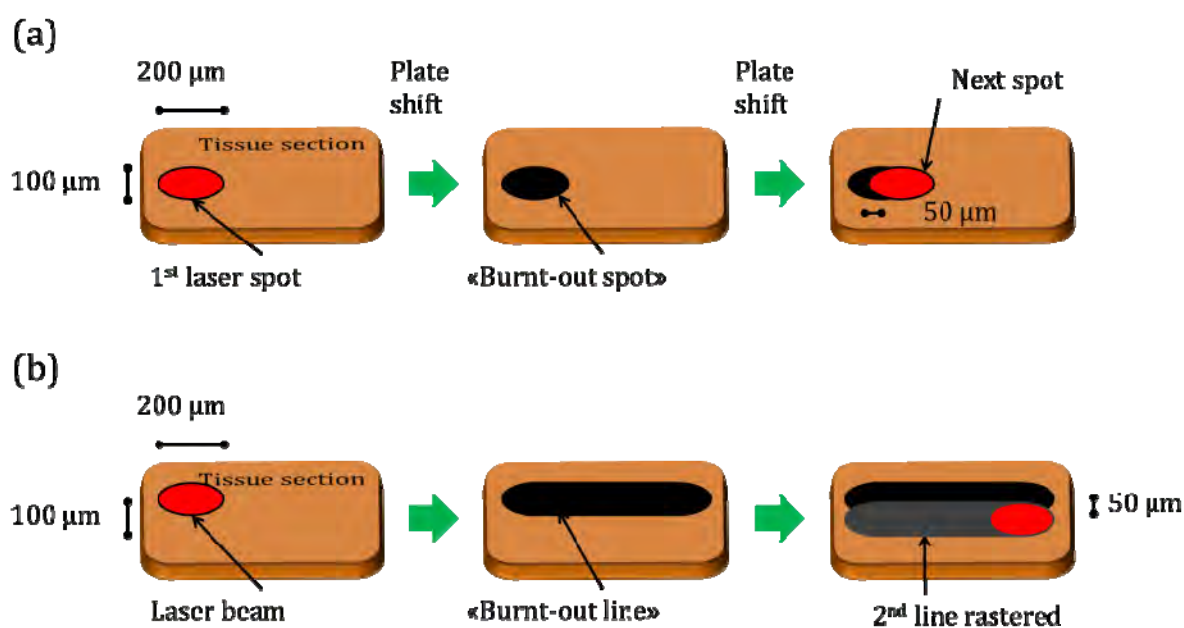


Figure I- 19. Oversampling strategy in (a) discrete mode and (b) rastering mode. Adapted and completed from ref.³²⁴

1.2.3.2. Microscope mode MS imaging: increasing the speed of data generation and acquisition

The introduction of ion microscope matrix-assisted laser desorption/ionization (MALDI) mass spectrometers is a major advance in the field of MS imaging. Microscope mode MSI enables to correlate mass spectral data with high spatial resolution down to the μm -range in a single experiment.^{307, 328} While microprobe mode imaging consists in collecting mass spectra from area as small as possible to maintain high spatial resolution (see previous paragraph), the ion microscope technique enables fast imaging through the extraction of ions from a large area of the sampled surface (typically ca. $200 - 300\ \mu\text{m}$ in diameter). These extracted ions are then projected onto a position-sensitive detector with improved spatial resolution. One drawback of this approach is the lack of adequate identification and separation capabilities that result from the implementation of the microscope approach on a time-of-flight system. The microscope mode developed for mass spectrometry imaging requires a high performance detector to take full advantage of this concept. One of the

requirements to perform a microscope MS imaging experiment is to link the time-of-flight (TOF) signal (*i.e.* the recorded mass-to-charge m/z ratio) with the initial position of the ion desorbed and ejected in the optical transferred chain. This cannot be achieved with the existing detection systems based on micro-channel plates (MCP) followed by a phosphor screen and a charge coupled device (CDD) camera or a resistive anode or delay-line detector, as different molecular masses cannot be recorded in one experiment. Active pixel detectors have been recently introduced as promising candidate to achieve high resolution and high throughput imaging experiments.³²⁹⁻³³² It was demonstrated that the time- and position-sensitive Timepix detector enables novel detection capabilities for large biomolecules up to 78 kDa (e.g. oligomers of the protein ubiquitin) in time-of-flight (TOF) based MSI analytical strategies.³³³ Mass-resolved ion images from Timepix measurements of peptide and protein standards demonstrate the capability to return the mass-spectral as well as the localization information of biological relevant compounds. Finally, the capabilities of using the time and position sensitive pixelated detector for mass spectrometric imaging of biological tissue sections have been successfully demonstrated.³³⁴

1.2.3.3. Desorption and ionization techniques used for MS imaging

The extraction/desorption and ionization of the analytes is achieved through exposure of the surface to a laser beam (e.g. LDI, MALDI, laser ablation), a primary ion beam (e.g. SIMS) or a charged droplet flux (e.g. DESI). Although MALDI is the most widely used ionization technique for MS imaging so far, development of ionization sources is still growing, and particularly, over recent years, an increasing interest for mass spectrometry imaging under ambient conditions has emerged.^{206, 335-338}

As the goal of this thesis is to extend the use of MS imaging to new applications, a concise review of the surface sampling and ionization techniques used in forensic toxicology and plant biology is presented Chapter 2 of this thesis manuscript.

1.2.4. Mass spectrometry-based strategies for the identification and targeted analysis of molecular species

1.2.4.1. High mass resolving power and high mass accuracy mass analyzers

a) Time-of-flight mass analyzers

The ability to accurately identify new molecular species is one of the key challenges in MS imaging for the identification (ID) of new biomarkers, or confirm the identity of targeted compounds. The majority of MALDI-MS instruments used for imaging are based on time-of-flight (TOF) mass analyzer. TOF-MS offers benefits of high mass range and high throughput, and latest developments have largely contributed to increase its performance in terms of mass resolving power and mass accuracy required for peaks ID. Particularly, the introduction of orthogonal acceleration geometry (oa-TOF) allows for hybridization

between TOF and quadrupole mass analyzers, i.e. QqTOF have been demonstrated particularly suited for the analysis of small molecules, with mass resolving power up to 50'000 and with MS/MS ability.^{144, 190, 193, 285, 339-341} The performances of the TOF/TOF analyzers have also been demonstrated, where the first TOF is used to select a precursor ion for fragmentation and the second TOF used for the rapid and parallel analysis of fragments.^{139, 234, 342-343}

b) Fourier transform mass analyzers

Fourier transform mass analyzers includes two types of mass analyzers: i) Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers, which determine the m/z of an ion from its ion cyclotron resonance frequency upon a static magnetic field; ii) Fourier transform orbitrap mass analyzers, which determine the m/z of an ion from its oscillation frequency (i.e. from axial oscillations after being trapped in between the outer and central electrodes) upon electrostatic field in the trap.³⁰⁴ Because the mass measurement is independent of the kinetic energy distribution of the ions unlike TOF-MS measurements, FT-MS provide a significantly higher accuracy than a TOF analyzer.

Although FT-ICR and orbitrap mass analyzers have the highest resolving power and better mass accuracy so far (i.e. > 100'000 and < ppm, respectively) and allows for multistage MS/MS capabilities,³⁴⁴ FT-ICR is not routinely used for high throughput imaging due to the long in-cell accumulation required. The applications in the imaging field are mainly limited to direct profiling from tissue section, or at least to image limited area, but not to image whole-body rat section. Endogenous compounds with the same nominal mass were successfully separated and the MS images were generated with a bin size down to $\Delta m/z = 0.005$ u.^{145, 168, 309-310, 327, 343-345} The high mass resolution MS imaging approach have enabled to reveal new features that were unresolved with lower resolution experiments. An example is given with the imaging of peptides in the rat brain using MALDI-FTICR mass spectrometry.³⁴⁴ The hybridization of FT-MS and linear ion-trap quadrupole has considerably revolutionized the performance of high resolution MS imaging approaches,³⁴⁶ and also the combination with atmospheric pressure such as AP-MALDI³⁴⁷⁻³⁴⁸, IR-MALDESI³²⁷ or a liquid extraction-based surface sampling.²⁴⁹

1.2.4.2. Tandem mass spectrometry detection

Despite their low mass resolution, quadrupole and ion trap mass analyzers are suited for targeted imaging of small molecules and offer unique capabilities of performing selective tandem mass spectrometry experiments. They provide additional MS information on the structural identification of compounds, by the use of MS/MS or MS³ experiments.³⁴⁹⁻³⁵² Specific reaction for targeted compounds can also be monitored *via* the selected reaction monitoring (SRM) mode, which provides so far the highest selectivity and sensitivity and allows for fast imaging.^{143, 191, 230, 257} One of the advantages of triple quadrupole over ion trap

is the absence of space charge effect. This is crucial when such mass analyzers are used in combination with sources that generate extensive chemical background (e.g. MALDI). If the performances of the trap can be improved by automatic gain control techniques, it results reduced duty cycle and reduced ability to profile very fast signal, which can be done with QqQ mass analyzers.

In 2009, Hopfgartner *et al.* introduced a platform based on a triple quadrupole linear ion trap (QqQ_{LIT}) mass analyzer fitted with a MALDI source for targeting drugs and imaging the distribution in a complete a whole-body rat section in less than 15 minutes. This platform offers the unique capability of combining a 1 kHz repetition rate laser for rapid data generation and fast SRM acquisition (see Chapter 3, Part 1 of this thesis manuscript for the details of this platform). This platform has been selected for the mapping and confirmatory analysis of tropane alkaloids in *E.coca* leaves in plant biology and also for the rapid detection of drugs of abuse and some of their metabolites in forensic toxicology to investigate postmortem tissue and intact hair samples (see Chapter 3).

1.2.4.3. Ion mobility

The interest for ion mobility in MS imaging has considerably increased over the last few years. The advantages of implementing a post-ionization gas-phase separation technique in an MSI workflow and the applications are detailed Chapter 4 of this thesis manuscript.

Briefly, one of the strengths of IMS is its ability to differentiate analytes that are isobaric but with different structures and also to discriminate ions of interest from interfering biological matrix ions.³⁵³ IMS has proven suitable for the analysis of various analytes, from small drugs,²⁸⁵ carbohydrates³⁵⁴ or lipids³⁵⁵⁻³⁵⁸ to peptides^{134, 158, 247, 359} and MDa protein and protein complexes.^{162, 360-363}

Table I- 9 gives an overview of commercially available instrument developed for MALDI-MS imaging, along with their main features.

Table I- 9. Overview of commercially available instrument developed for MALDI-MS imaging and features. Instruments *in italic* belong to “old generation” instruments. Resolution st*1 = mechanical resolution limited by sample stage positioning or software. Resolution la*2 = approximate laser beam diameter on sample. NB: Bruker smartbeam lasers are actually Nd:YAG lasers modified by a modulator to provide structured beam profiles.³²² Completed from ref. www.maldi-msi.org.

Instrument	Company	Analyzer	Laser	Mass Range	Resolution (st*1, la*2)	Software	Data Format
Flashquant	AB Sciex	QqQLIT	Nd-YAG 1 kHz	0 - 2400 Da	30 μ m 100 μ m	oMALDI Server 5.1, TissueView, Biomap	Analyze
5800 MALDI TOF/TOF™ Analyzer	AB Sciex	TOF/TOF	Nd-YAG 1 kHz	full	1 μ m	4800 Img, TissueView	Analyze
4800 Plus MALDI TOF/TOF™ Analyzer	AB Sciex	TOF/TOF	Nd-YAG 200 Hz	full	1 μ m 50 μ m	4800 Img, TissueView	Analyze
QSTAR® Elite System	AB Sciex	QqTOF	Nd-YAG 1 kHz	40 to 40 kDa	30 μ m 150 μ m	oMALDI Server 5.1, TissueView	Wiff, Analyze, imzML
QSTAR® Pulsar i System	Applied Biosystems / MDS Sciex	QqTOF	Nd-YAG 1 kHz	40 to 40 kDa	30 μ m 150 μ m	oMALDI Server 5.1, TissueView	Wiff, Analyze
QSTAR® XL System	Applied Biosystems / MDS Sciex	QqTOF	Nd-YAG 1 kHz	40 to 40 kDa	30 μ m 150 μ m	oMALDI Server 5.1, TissueView	Wiff, Analyze
4700 Proteomics Analyzer	Applied Biosystems / MDS Sciex	TOF-TOF	Nd-YAG 200 Hz	full	1 μ m 50 μ m	4700 Img, TissueView	Analyze
Voyager DE™ STR Workstation	Applied Biosystems / MDS Sciex	TOF	N2 20 Hz	full	1.6 μ m 100 μ m	MMSIT, BioMap	Analyze
Voyager DE™ PRO Workstation	Applied Biosystems / MDS Sciex	TOF	N2 20 Hz	full	1.6 μ m 100 μ m	MMSIT, BioMap	Analyze
ultrafleXtreme	Bruker	TOF/TOF	smartbeam- II 1 kHz	full	5 μ m 10 μ m	flexImaging, CreateTarget, AnalyzeThis!, BioMap	Xmass, XML
Solarix MI	Bruker	hybrid FTMS	smartbeam- II 1 kHz	100 - 10 kDa	5 μ m 20 μ m	flexImaging, BioMap	Xmass, XML
autoflex III, ultraflex III	Bruker	TOF-TOF	smartbeam 200 Hz	full	5 μ m 10 μ m-30 μ m	flexImaging, CreateTarget, AnalyzeThis!, BioMap	Analyze

Table I- 9. Continued.

Instrument	Company	Analyzer	Laser	Mass Range	Resolution (st*1, la*2)	Software	Data Format
JMS-S3000 SpiralTOF™	JEOL	TOF/TOF	Nd:YLF 250Hz (opt. 1kHz)	to 500 kDa (linear) to 30kDa (spiral)	10 μm 30 μm	msTornado, BioMap	imzML
iMScope imaging mass microscope	Shimadzu	ion trap TOF	Nd-YAG 1 kHz	n.a.	5 μm	n.a.	n.a.
MALDI-7090	Shimadzu	TOF/TOF	2 kHz	n.a.	n.a.	MALDI Solutions software	n.a.
AXIMA QIT™	Shimadzu	ion trap TOF	N2	to 20 kDa	10 μm 100 μm	Axima2Analyze	Analyze
AXIMA Assurance	Shimadzu	TOF	N2	to 500 kDa (linear)	10 μm 100 μm	Axima2Analyze	Analyze
AXIMA Confidence AXIMA Performance	Shimadzu	TOF TOF/TOF	N2 50 Hz	to 500 kDa (linear) to 80 kDa (reflectron)	10 μm 100 μm	Axima2Analyze	Analyze
MALDI LTQ XL	Thermo Fisher Scientific	ion trap	N2 60 Hz	50 – 4000 Da	1 μm 60 μm	LTQ Tune, ImageQuest	RAW, imzML
MALDI LTQ Orbitrap Discovery	Thermo Fisher Scientific	ion trap – Orbitrap hybrid	N2 60 Hz	50 – 4000 Da	1 μm 60 μm	LTQ Tune, ImageQuest	RAW. imzML
MALDI LTQ Orbitrap XL	Thermo Fisher Scientific	ion trap – Orbitrap hybrid	N2 60 Hz	50 – 4000 Da	1 μm 60 μm	LTQ Tune, ImageQuest	RAW, imzML
MALDI SYNAPT G2 HDMS	Waters	Q-IMS- oaTOF	Nd-YAG 1 kHz	to 40 kDa	3.5 μm 60/200 μm	MassLynx, BioMap	XML
MALDI SYNAPT G2 MS	Waters	Q-TWave- oaTOF	Nd-YAG 1 kHz	to 40 kDa	3.5 μm 60/200 μm	MassLynx, BioMap	XML
MALDI Q-ToF Premier	Waters	Qq-oaTOF	Nd-YAG 200 Hz	to 40 kDa	25 μm 100 μm	MassLynx, BioMap	XML
MALDI micro MX	Waters	TOF	N2 20 Hz	full	25 μm 200 μm	MassLynx, BioMap	XML

1.2.5. Software tools for data processing and image visualization

1.2.5.1. Software for MS imaging

A single MS imaging experiments can create data sets from many tens to hundreds gigabytes and interpretation of molecular profiles is challenging. The mostly used platforms provided by the vendors are: **TissueView** (AB Sciex), **flexImaging** (Bruker) and **ImageQuest** (Thermo), but several software programs have been introduced to process and visualize MALDI MSI data (Table I- 10). Additional software package are also available to convert proprietary data formats for importation in one of these software (e.g. *Axima2Analyze* convert data from Shimadzu instruments to be used in **BioMap**; *MSImage View* converts data acquired with the FlashQuant instrument [.wiff data from AB Sciex] into jpeg, Analyze (BioMap) or imzML format). On the prototype version of the FlashQuant software used during this PhD work, a dedicated script has been written by E. Duchoslav (AB Sciex) to convert .wiff raw data into .img format compatible either with BioMap or TissueView software for visualizing the data). So far, two softwares enable quantification:

i) **Quantinetix** (ImaBiotech, France), is the first commercial software developed to assist quantification in MS imaging.³⁶⁴ Quantification can be performed following different quantification strategies such as the use of standards spotted onto tissue, isotopically labeled standards or based on the calculation of tissue extinction coefficient to quantitate the amount of analyte in the tissue;¹⁶⁹

ii) and **MALDI Tools**, developed by Andren and co-workers, assists labeled normalization and quantification of drugs and neuropeptides directly in tissue sections. The concentration of a specific analyte in a defined region of interest on a tissue section can be calculated by correlating its value to a standard curve of the analyte on control tissue. The ion of interest is selected and the spot area for the selected ion, the density and the thickness of the tissue section can be defined. Standard curves of analytes of interest are displayed graphically and the linear response, regression, intercept and correlation coefficient are calculated automatically.³⁶⁵

1.2.5.2. Data sharing: imzML format

In order to allow the flexible and efficient exchange of MS imaging data between different instruments and data analysis software, the data format imzML has been recently introduced.³⁰⁹ The objective is to make imzML format supported by a growing number of software tools, so that the users will be no longer limited to proprietary software, but are able to use the processing software best suited for a specific question or application. MS imaging data from different instruments can be converted to imzML and displayed with identical parameters in one software package for easier comparison,³⁶⁶ and open source converter tools are available (i.e. imzMLConverter to convert mzML to imzML³⁶⁷).

Table I- 10. Overview of software developed for visualization and analysis of MS imaging data. References: maldi-msi.org. Data Cube Explorer.³⁶⁸ Quantinetix.³⁶⁴ MALDI Tools.³⁶⁵ MITICS.³⁶⁹ Create Target/Analyze this!³⁷⁰ MSiReader.³⁷¹ MALDIVision.³⁷² *TIC: total ion count; ROI: region of interest.

Features	BioMap	TissueView	Data Cube Explorer (DCE)	FlexImaging	Quantinetix
Autor, Copyright	Novartis (M. Raush, M. Stoeckli)	Novartis AB Sciex	FOM Institute AMOLF (The Netherlands)	Bruker	ImaBiotech (France)
Type	Image reconstruction	Image reconstruction	Image reconstruction	Acquisition/ Image reconstruction	Image reconstruction / Quantitation
Compatibility with instruments	AB Sciex (MALDI-TOF, 4700-4800 MALDI-TOF/TOF) Waters (MALDI Micro XM™)	AB Sciex (MALDI-TOF/TOF and STAR; conversion required to read data acquired from the FlashQuant)	-	Bruker (MALDI-TOF and MALDI-TOF/TOF)	Any type MALDI or LESA-generated image (TriVersa NanoMate) Bruker AB Sciex Thermo
Data format input	Analyze 7.5, .tiff, .img, .dicom, .png	.wiff raw data	Instrument format imzML	Instrument format, .img	Instrument format imzML
Output image format	.tiff, .jpeg, Analyze 7.5	.tiff, .jpeg, Analyze 7.5		.bmp, .jpeg, .jpg, .tif, .tiff	
Spectral processing tool, baseline subtraction and baseline denoising	Yes	Yes	Yes	Yes	Yes
Normalization methods	TIC, Normalization with labeled compound enabled	TIC, Normalization with labeled compound enabled	n.a.	Yes (details unknown)	Normalization based on TIC, isotopically labeled standards, ion-suppression calculations
Selection of ROI and calculation of average spectrum	Enabled Coregistration possible / overlay MS spectra displayed from a selected area or averaged for each ROI	Enabled Coregistration possible / overlay MS spectra displayed from a selected area or averaged for each ROI	Yes, image-based and spectrum-based visualization of data	Unknown	Molecular overlay with multiple mass filters for a better view of molecular co-localization
3D visualization of data	Yes	Yes	Yes (with Visualization Toolkit)	Unknown	No
Quantitation enabled	No	No	No		Yes
Statistical tool integrated	No	No	No	Clinprotools (hierachical clustering, PCA)	No

Table I- 10. Continued.

Features	MALDI Tools	MITICS	MSiReader	MALDI Vision	ImageQuest	Imaging Creat Target AND Analyze This!
Autor, Copyright	Uppsala University (Sweden) Medical MS Research group	University of Lille (France) MALDI Imaging Team	North Carolina State University, Raleigh, NC, USA	PREMIER Biosoft	Thermo Scientific	
Type	Image reconstruction / Quantitation	Acquisition/ Image reconstruction	Image reconstruction	Image reconstruction / Data processing	Acquisition / Data conversion	Acquisition / Data conversion
Compatibility with instruments	Bruker	AB Sciex (Acquisition) Other platforms (BD MALDI- TOF, MALDI- TOF/TOF) (visualization)	AB Sciex, Bruker, Thermo Scientific, Agilent, Waters, Shimadzu, after conversion of data in appropriate format if needed		Thermo Scientific (MALDI LTQ XL, LTQ Orbitrap hybrid series)	Bruker (MALDI- TOF and MALDI- TOF/TOF)
Data format input	XMASS raw data	.xml	.imzml, Analyze 7.5, .ascii, .mzXML	Analyze 7.5 imzML	Instrument format	Instrument format
Outfput image format	XML	.bmp, .jpg, .jp2, .gif, .tif, .pict, .png	Unknown	imzML	Unknown	Analyze 7.5
Spectral processing tool, baseline substraction and baseline denoising	Yes	Yes	Yes	Yes	-	-
Normalization methods	TIC, Root mean square, median normalization and labeled normalization		Unknown	Yes	-	-
Selection of ROI and calculation of average spectrum		Not specified	Yes	Yes	-	-
3D visualization of data		No	No	Yes	-	-
Quantitation enabled	Yes	No			-	-
Statistical tool integrated	No	No	No	No	-	-

1.2.5.3. Bioinformatics and statistical tools

Profiling and imaging biological specimens using (MALDI) mass spectrometry has significant potential to contribute to our understanding and diagnosis of disease. The technique is efficient and high-throughput providing a wealth of data about the biological state of the sample from a very simple and direct experiment. However, in order for these techniques to be put to use for clinical purposes, the approaches used to preprocess and analyze the data must improve.^{109, 373}

For biomarker discovery based on (MALDI) MS imaging, automated and reliable computational methods are required to analyze dataset containing large number of different molecules detected, over a broad mass range in a single experiment.^{234, 374-375} Preprocessing of the (MALDI) spectra is of utmost importance to remove sources of variation or background peaks other than the biologically relevant peaks³⁷⁶⁻³⁷⁷ in the spectra and consists in baseline correction, alignment and calibration, smoothing and peak picking. Among the statistical tools used to reduce dataset, **data mining** is shown to reduce the number of biomarkers candidates.³⁷⁸ **Principal component analysis** (PCA), in combination with data binning or other reduction algorithms, is the most common unsupervised data analysis approach and is used as a dimensionality reduction method, by grouping together peaks whose intensities are correlated in certain areas of tissue, prior to clustering and spatial segmentation.³⁷⁹⁻³⁸⁰ To illustrate, this approach has been used to discover different biomarkers in starvation-induced fatty livers and normal livers.³⁸¹ In combination with **hierarchical clustering**, discrimination could be established between gastric cancer and non-neoplastic mucosa tissue sections.³⁸² Other studies have reported the discovery of biomarkers using MALDI-IMS in Parkinson's disease³⁸³ or in the histopathologic examination of atherosclerotic lesions.³⁸⁴

1.2.5.4. Three-dimensional (3D) visualization of images

The additional spatial dimension to 2D MS imaging enables the interrogation of analyte expression patterns in an entire volume. This allows for delivering contextual information to 2D images and reducing the possibility of neglecting small anatomical structures.³⁸⁵⁻³⁸⁶

3D MS imaging can be created in two fashions: i) **sequential registration of multiple tissue sections** (i.e. 2D) and combination in a single visual representation (i.e. 3D); this is achievable with ionization sources such as MALDI³⁸⁷ or DESI;³⁸⁸ ii) **depth profiling** by high resolution secondary ion mass spectrometry,^{308, 389-390} laser ablation ESI (LAESI)³⁹¹ and laser ablation coupled to a flowing atmospheric pressure afterglow (LA-FAPA).³⁹²

The accurate coregistration of 2D images in a 3D volume is by far the most critical step. In addition, comparison with other modalities (e.g. fluorescence microscopy, MRI³⁷⁸) can be required. Chughtai *et al.* have reported a co-registration technique to compare MSI and fluorescence microscopy which consists in using fiducial markers that possess a combination of optical and molecular properties, which result in a clear mass spectrometric

signature. These fiducial markers used as reference on each tissue section allow for accurate coregistration and 3-dimensional reconstruction of serial histological and fluorescent microscopic images with MSI images of thin tissue sections from a breast tumor model.²⁰²

3D MSI have been successfully used in the field of microbiology to characterize microbial metabolic exchange^{308, 387} and also for 3D single cell imaging,³⁹³ drug and pharmaceutical analysis, metabolites and lipids imaging, and imaging proteins and peptides in small brain structures such as the substantia nigra.³⁹⁴

1.2.6. Conclusion and perspectives

MS imaging was presented in the first part of the introduction as one of the keys tools in biomedical research, in particular in the drug discovery and development and/or to identify potential disease biomarker, such as cancer biomarkers, directly from tissue sections. By allowing parallel analyses of multiple molecular species in a single run, MSI has considerably increased the analysis throughput in clinical proteomics studies or in drug discovery. One of the strength of MSI is its ability to visualize these molecular species at the surface of biological samples, without any labeling, and thus without any prior knowledge of the sample composition. This leads to establish MS imaging as an indispensable tool for studying biological systems. The developments achieved so far to push forward the limits in terms of ultra-high spatial resolution, high throughput, and high sensitivity offer a large panel of analytical possibilities in the field. This supports to answer biologically relevant questions, regarding a wide variety of biological samples and molecular species, from low molecular weight to high mass proteins. Now, the researchers can make use of high mass resolution, high mass accuracy, tandem MS strategies or even ion mobility to identify molecular species detected on biomedical tissue surfaces. One of the research areas that has benefit from a particular attention is the development of ionization sources. Although SIMS is by far the favorite method for single cell analysis and high resolution imaging, advanced developments in instrumentation have enabled to increase the spatial resolution. In laser-based ionization approaches, in particular microprobe MALDI-MSI, 5 μm is the current applicable limit. If MALDI remains the most widely used ionization technique in MSI, the last years have seen the emerging field of ambient ionization techniques broadening the analytical possibilities. This is for instance the applications to the analysis of drugs and their metabolites in plant biology and toxicological and forensic sciences (see Chapter 2 of this thesis). The development of intuitive and comprehensive software to process the large amount of data generated, including statistical tools and the possibility to perform quantification in a quasi-automated way and high throughput, is one of the challenges for the next developments to be done to emphasize the value of MSI in protein/cancer biomarker discovery. Although MS imaging is now well established as a qualitative tool, its use as quantitative tool is still controversial. Another challenge is thus to perform direct quantification from tissue sections, and this is the main concern of the Chapter 5 of this thesis.

CHAPTER 1.

Imaging in biomedical research and the role of MSI

Part 3 – Aims of this thesis



The main objective of this PhD thesis is to identify and develop analytical platforms for the direct detection and structural confirmation of low molecular weight compounds (i.e. pharmaceuticals, drugs of abuse and their metabolites) in biological tissues. This work aims at spreading their use to new applications such as the analysis of drugs and their metabolites in **plant biology** (i.e. in intact leave samples) and **forensic toxicology** (i.e. in hair and postmortem human samples). For a review of the state-of-the-art of MSI in this two fields, see **Chapter 2** of this thesis manuscript.

For that purpose, two platforms based on triple quadrupole linear ion trap mass analyzers are mainly used and developed:

- a) a 4000 QTRAP mass analyzer fitted with a high repetition rate **matrix-assisted laser desorption/ionization** (MALDI) source, operated at intermediate pressure conditions. The platform has been exploited for its ability to generate and acquire data in a rapid fashion, its high sensitivity and its high selectivity. These criteria are of utmost importance when analyzing complex samples such as tissue sections, and when no separation technique (e.g. no liquid/gas-phase chromatography nor thin-layer chromatography) precedes the MS analysis (**Chapter 3**);
- b) the next generation of QTRAP mass analyzer, i.e. the QTRAP 5500, equipped with a **differential ion mobility** cell in front (i.e. the SelexION technology lately developed by AB Sciex). This unique platform has been identified as a powerful tool to be used in combination with a chip-based nano-electrospray ionization source operated at ambient temperature, the TriVersa NanoMate, which is capable of liquid extraction-based surface sampling (i.e. **liquid extraction surface analysis**, LESA). The development of this platform focuses on the analysis of isomers and their separation in the gas-phase, prior to MS detection (**Chapter 4**).

Through the development and the applications of these two platforms, this thesis highlights the experimental parameters that have to be considered to generate MS images of quality (**Chapter 3 - Parts 3 and 4a; Chapter 5 - Part 2**). This thesis emphasizes how multimodality imaging techniques, namely MALDI, LESA and/or SIMS (secondary ion mass spectrometry) can be employed to respond a specific analytical question (**Chapter 3 - Parts 3 and 4b**).

Some issues related to the direct quantification of drugs and their metabolites direct from tissue sections by MS imaging are also addressed. After identifying and discussing the main challenges inherent to direct quantification from tissue sections, this thesis presents the first steps toward a concise methodology for the generation of standards for quantitative MSI and discusses the different approaches developed so far (**Chapter 5 - Parts 1 and 3**).

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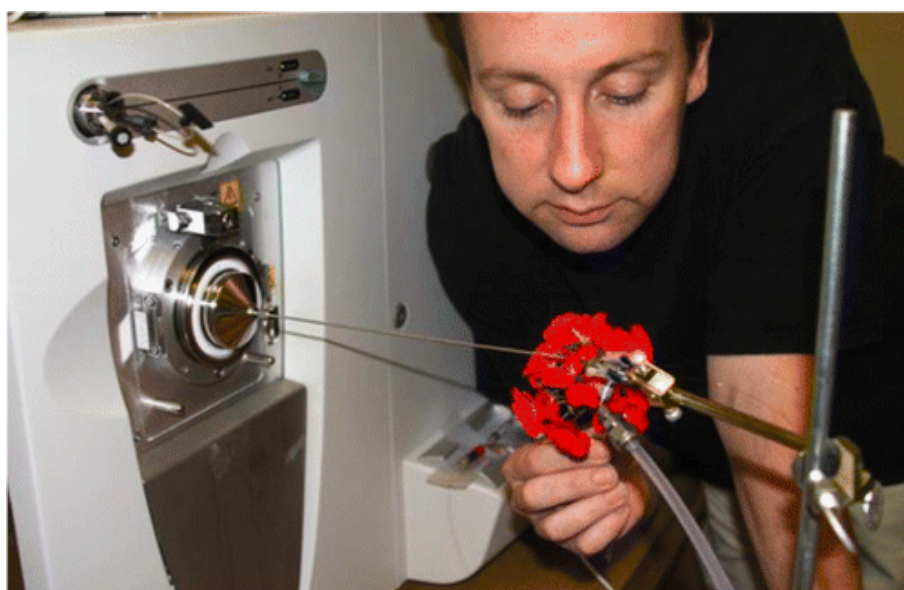
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2

CHAPTER 2. Molecular imaging by mass spectrometry: applications in forensic toxicology and plant biology – *A review*



Review adapted and completed from:

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2.1. Introduction

Imaging mass spectrometry-based techniques are label-free and rely on the desorption of molecules present at the surface of a solid sample, preferably flat. High spatial resolution and intact integrity of the sample are all the features required to get high quality image, full of precious information. The interest for molecular imaging based on mass spectrometry (i.e. MSI, for **mass spectrometric imaging**) has gained of interest in the past 20 years in various fields including **forensic toxicology** and more recently, **plant biology**.

The main advantage provided by these label-free techniques is the conservation of the spatial integrity of the sample and thus keeps intact spatial information. MS imaging was previously developed as a **direct surface analysis** to map pharmaceuticals in whole body rat sections and characterize the distribution of the parent drug and its metabolites within the sample, by recording the mass-to-charge ratio (m/z) signal of molecules of interest at each spatial location, defined by the pixel.¹⁻⁵

The first part of this article focuses on **secondary ion mass spectrometry** (SIMS) and **matrix-assisted laser desorption/ionization** (MALDI) MS imaging, as well as on the rapidly developing field of ambient MS techniques as alternative desorption/ionization techniques.⁶⁻¹¹ These techniques have the benefit of direct analysis of surfaces without pre-treatment, which considerably speeds up analysis and reduces sample handling. Among these techniques, the first developed has been **desorption electrospray ionization** (DESI), rapidly followed by many others, including **direct analysis in real time** (DART), **plasma assisted desorption ionization** (PADI), **extractive electrospray surface ionization** (EESI), **laser ablation electrospray ionization** (LAESI), just to name a few.

Their main applications in forensic toxicology are reviewed as well as the advantages provided in terms of sample preparation over approaches routinely used in toxicological laboratories. The second part of the application depicts the used of MS imaging in the field of plant biology. Although the first aim of this review is to highlight the applications of molecular MS imaging in both areas, i.e. the term “imaging” involving the generation of an image as a result of the experiment, in the absence of representative examples in the literature, some examples of the use of these techniques as “direct surface analysis” will be shown.

2.2. Secondary ion mass spectrometry (SIMS)

SIMS was introduced in 1977 by Benninghoven¹² and has been shown as a powerful technique for molecular surface analysis. The ionization mechanism occurs under ultra-high vacuum where the surface of the sample is sputtered with a pulsed high-energy (5 to 25 keV) primary ion beam (e.g. Cs^+ , O^- , Ga^+ , and cluster or polyatomic ion sources such as C_{60}^+ , Au_n^+ and Bi_n^+).¹³ The resulting secondary atomic or molecular ions are ejected and collected with high-voltage extraction lenses (Figure II- 1). The focused ion beam enables ultra-high resolution images for atomic ions and low molecular mass fragments but the relevant ion

yield from pixels in the sub-1 μm region is very limited since less than 1% of the surface is removed during the analysis.

SIMS is suitable for elemental and low molecular weight compounds analysis. A significant lack of sensitivity of the mass range above m/z 2000 is observed due to in-source fragmentation of complex molecules.¹⁴ A matrix, like in MALDI, can be applied onto the sample to prevent extensive fragmentation and increase the ionization efficiency (i.e. matrix-enhanced secondary ion mass spectrometry, ME-SIMS).¹⁵ It is also possible to cover the surface with a thin layer of metal such as gold (i.e. metal-assisted SIMS, or Met-SIMS)¹⁶⁻¹⁷ to increase the conductivity of the sample, and thus increase the sensitivity, which is particularly suitable in combination with ToF analyzers, where the ions are initially all accelerated by an electrical field prior their entrance into the mass analyzer. Except for these two exceptions (i.e ME-SIMS and Met-SIMS), no particular sample preparation is required. SIMS is also surface sensitive and only the first mono-layer or less is removed during sputtering. This feature allows depth profiling with high resolution within the μm range and below.

2.3. Laser-based desorption/ionization techniques

2.3.1. Laser desorption/ionization (LDI)

Introduced in the 1970s, laser desorption ionization (LDI), i.e. MALDI without the need of a UV-absorbing matrix, has been performed on non-volatile analytes up to 1-2 kDa, on the condition that the analytes possesses a UV-absorbing chromophore moiety. The main advantage of LDI over MALDI is that no matrix is required, which prevents in a MS imaging experiment the potential delocalization of analyte during the application of a wet matrix on the top of the sample. Although LDI is popular in forensics, the most of the studies carried out in biological samples, including plant material, have revealed the presence of highly abundant species, such as endogenous metabolite species in plants (see § 2.2.a.).¹⁸⁻¹⁹

2.3.2. Matrix-assisted laser desorption/ionization (MALDI)

Karas *et al.* introduced MALDI under its current form in the late 1980s.²⁰ It is the most widely used ionization technique for MS imaging and can be operated either at atmospheric pressure or under vacuum condition. MALDI is based on the homogeneous application of a matrix in excess on the top of the sample, resulting in the co-crystallization of the matrix and analytes. Matrix coating is critical and must keep intact the analyte localization within the sample. The matrix is usually a small organic compound (*e.g.* alpha-cyano-4-hydroxycinnamic acid, sinapinic acid, 2,5-dihydroxybenzoic acid) that plays an important role in the ionization process by absorbing the photon energy emitted by the laser (Figure II- 1). By using an appropriate matrix, MALDI-MS is suitable for characterizing a wide range of compounds, including large (*i.e.* proteins, peptides) and small molecules (*i.e.* pharmaceuticals, lipids) at low femtomole to attomole levels. Nowadays, most MS instruments can be used for MALDI MS imaging (see, for example: www.maldi-msi.org). Hybrid instruments such as triple quadrupole linear ion trap (QqQ_{LIT}), quadrupole-time-of-

flight (QqTOF), TOF/TOF, ion trap-TOF (QIT-TOF) or linear ion trap-orbitrap, offer several possibilities to perform MS and/or MS/MS imaging experiments. Accurate mass is attractive for selectivity however QqQ_{LIT} operated in selected reaction monitoring (SRM) mode remain essential for sensitive and fast detection. When comparing MS instruments performance, several aspects have to be considered in terms of resolution, selectivity, speed of acquisition and sensitivity. In all cases, the spatial resolution is dependent on the dispersion and the size of matrix crystals and on the laser beam characteristics. Recent developments in MS instrumentation enable rapid data generation with high repetition rate lasers (*i.e.* frequency up to 20 kHz), and focused laser beam to achieve a spatial resolution in the order of 10 – 50 μm .

2.3.3. Surface assisted laser desorption/ionization techniques (SALDI)

The first objective with the development of surface-assisted laser desorption/ionization (SALDI), defined as “matrix-free” techniques, was to overcome the issues encountered with “wet matrix”-assisted LDI techniques, *e.g.* inhomogeneity in the surface coating, extraction efficiency of the analyte from the biological material, co-crystallization of the matrix with analyte.

SALDI uses metal-, silicon- and carbon-based nanostructured materials as matrices, giving several related techniques, namely desorption/ionization on silicon (DIOS) or nanostructure initiator mass spectrometry (NIMS), colloidal graphite assisted LDI (GALDI)²¹ or colloidal silver assisted LDI,²¹⁻²² just to name of few. Nanoparticles have been proven useful matrices for the analysis of biomolecules (including proteins, peptides, small molecules), mainly because of their large surface area, strong absorption in the ultraviolet-near-infrared region, and ready functionalization which allows for concentration of the analyte. The nanoparticles can be released upon both ion or laser (*i.e.* with common N₂ or Nd:YAG lasers) irradiation.

As an example, NIMS, which is a further development of DIOS,²³⁻²⁴ was introduced by Siuzdak and co-workers in 2007,²⁵⁻²⁶ and utilizes nanostructured surfaces. These nanostructured surfaces consist in silicon surface (an efficient UV-absorbing semiconductor with a large surface area) composed of pores of *ca.* 10-20 nm in diameter to trap liquid 'initiator' materials. During the NIMS desorption/ionization, the porous silicon absorbs laser energy, which results in a rapid surface heating and vaporization of the trapped initiator. This is followed by desorption/ionization of intact molecules adsorbed on the surface of the initiator (Figure II- 1). NIMS initiators (such as lauric acid, polysiloxanes, siloxanes and silanes) are fundamentally different from MALDI matrices because the initiators do not absorb UV energy, most do not ionize, and the analytes are not co-crystallized with the initiator. Because there is no ionization of the initiator, low molecular weight analytes can be examined with improved signal-to-noise (S/N) ratio and minimal background ion interferences in the low *m/z* range. This considerably extended the observable mass range compared to SIMS for metabolites and drugs studies.²⁷⁻²⁸ The lateral resolution achieved is limited by the laser beam, and as be shown as good as 15-20 μm , which is better than the common resolution achieved with MALDI. For the analysis of biofluids, human saliva or

urine samples are directly spotted on the NIMS surface without any further sample preparation.²⁹

For tissue analysis, 3–5 μm -thickness sections are simply collected on the NIMS surface.²⁸⁻³⁰ However, unlike other existing MSI experiments where analytes present at the surface of the tissue are desorbed and ionized, NIMS only allows for imaging analytes at the tissue/surface interface. For that purpose, a two-steps ablation is required: i) the surface tissue is ablated with high laser energies (*ca.* $0.4 \text{ J}\cdot\text{cm}^{-2}$ / pulse) and ii) the tissue/surface interface is then analyzed with lower-energy laser irradiation (*ca.* $0.01 \text{ J}\cdot\text{cm}^{-2}$ / pulse) for mass spectral imaging. In that experimental setup, the lateral resolution achieved is of $50 \mu\text{m}$.²⁵

2.3.4. Laser-ablation inductively coupled plasma-MS (LA-ICP-MS)

Established as a powerful tool to investigate and quantify elements and isotopes directly in the solid samples, LA-ICP-MS has recently emerged in the field of MS imaging applied to life sciences,³¹⁻³³ including plant biology,³⁴ and also knows few applications in forensics, such as (trace) element analysis of car paints.³⁵ LA-ICP-MS utilizes a focused laser beam (e.g. such as the solid-state Nd:YAG laser used in MALDI, which can be focused down to $5 \mu\text{m}$ in spot size) to raster the surface of the sample, which induce the expansion of the material in a plume. The laser induced plume is then transported by a carrier gas (i.e. He or Ar) to the inductively coupled plasma (ICP) where ionization of the ablated material occurs, prior the introduction of the ionized molecules to the mass analyzer (Figure II- 1). The ionization efficiency in LA-ICP-MS is very high, which explain that the detection limits can be as low as trace levels.

2.4. Electrospray-based Desorption/Ionization Techniques

2.4.1. Desorption Electrospray Ionization (DESI)

DESI was developed by Cooks and co-workers in 2004 and is one of the continuously evolving desorption and ionization ambient technique.³⁶ Commonly, charged droplets are generated by a pneumatically assisted electrospray interface and are directed toward the flat sample at a defined angle (i.e. usually $45\text{--}60^\circ$). This creates a thin liquid film at the surface of the sample, and the molecules present are extracted. A second generation of charged droplets is then produced and suctioned into an extended atmospheric pressure ion transfer capillary that transports the ions into the vacuum region of the mass analyzer where they are analyzed. The ionization of the desorbed molecules from the secondary droplets is dependent on the solvent composition and pH of the solvent as seen for ESI, and ESI-like ions are produced.³⁷

Except the sample preparation common to all the direct surface analysis techniques (i.e. cryosectioning for tissue imaging), DESI does not require any sample preparation. Some studies have reported the addition of surfactant in the solvent used for extraction leads to the improvement in ion intensity. This is mainly attributed to the effect of surface tension in producing smaller spray droplets, covering a larger surface area.³⁸ DESI-MS is suitable for the analysis of small and large molecules with moderate sensitivity compared to MALDI-MS, but allows the analysis of a broader range of analytes. However, due to the large surface area

sampled by the electrospray beam, the imaging spatial resolution achieved with DESI is above several hundred μm (i.e. 0.25–1 mm). However, there is actually no need in forensics investigations to reach such spatial resolution performance for the samples to be analyzed. Reactive DESI has been recently introduced to allow solution-phase reactions. It consists in incorporating specific reagents in the solvent system to derivatize the analytes and thus enhance the selectivity and sensitivity, as it has been shown for cholesterol for instance.³⁹

2.4.2. Nanospray desorption electrospray ionization (*nano-DESI*)

Recent developments have led to the improvement of the spatial resolution often criticized with the DESI approach. The nano-DESI approach uses two fused-silica capillaries attached at a solvent bridge formed on the sample surface.⁴⁰⁻⁴¹ One capillary called the “supply” pumps the solvent onto the surface at an angle while the self-aspirating “nanospray” capillary (i.d. 50 μm) is positioned such that it is at an angle or orthogonal to the surface and positioned near the MS inlet. By adjusting the solvent flow rates from the supply capillary can be adjusted so that the effective sampled spot size can be modified (i.e. ca. 100–300 μm). However, recent MSI study has shown the ability to sample surface areas at a spatial resolution better than 12 μm .⁴²

2.4.3. Sonic spray ionization (*SSI*)

Unlike other techniques using a high voltage or heating, sonic spray ionization (SSI) is a desorption/ionization technique using a high-velocity spray⁴³ to generate small droplets, from polar solutions (typically methanol/water). The instrumentation is quite simple and only requires a supersonic nebulizing gas flow (i.e. using nitrogen or air as gas at ca. 200 psi) coaxial to the fused-silica capillary, where the spray comes from. Charged droplets and consequently gaseous ions are produced at atmospheric pressure due to statistical (unbalanced) charge distribution during droplet formation in the supersonic pneumatic spray. SSI is therefore gentler than ESI, producing ions of lower internal energy and lower charge states. Other advantages of the technique include the lack of electrochemical or oxidation processes and the low charge density of the droplets, which may decrease chemical noise coming from the solvent. Desorption of analytes from the surface is believed to follow similar mechanisms as DESI.⁹

Easy ambient sonic spray ionization (EASI), previously referred to as desorption sonic spray ionization (DeSSI), is a variant of SSI.⁴³⁻⁴⁵ EASI utilizes a high-velocity spray to form small droplets directed at the sample surface, which dissolves the analytes, and transport secondary droplets into the mass analyzer. EASI has been employed in forensics for various applications such as ink ageing or counterfeit banknote analysis.⁴⁶⁻⁴⁷

2.4.4. Extractive electrospray ionization (*EESI*)

EESI, developed by Zenobi and co-workers,⁴⁸⁻⁵¹ is a gentle ambient ionization method where a gas is allowed to flow over the sample to desorb molecules and then the gas is directly inserted into the electrospray region of ESI-MS to ionize the molecules. A variant of the EESI

consists in using a nitrogen-flow instead of solvent, into the ESI spray, i.e. neutral desorption (ND-) EESI. Due to its great tolerance to complex matrices and its ability for on-line measurements, (ND-) EESI has a broad range of potential applications such as in forensics (e.g. for analyzing trace amounts of explosives on human skin) or food control and environmental analysis.

2.5. Hybrid desorption/ionization mechanisms

2.5.1. Laser ablation electrospray ionization (LAESI)

Laser ablation electrospray ionization (LAESI), which in principle is similar to IR-LADESI,⁵² is a hybrid ambient ionization technique introduced in 2007. LEASI involves both laser desorption of the analyte followed by its ionization in the course of ablated material with charged droplets produced by ESI.⁵³⁻⁵⁵ LAESI uses a focused mid-IR laser beam operated at the wavelength of 2.94 μm to sample material directly from tissue based on the strong absorption of water at this wavelength. As a consequence, and because the principle phenomenon occurring in LEASI is the excitation of the OH vibrations of water molecules in the sample (i.e. we can say that the matrix acts as an intrinsic matrix in that case), LEASI is particularly well-suited for the investigation of any water-containing specimens. But this is at the same time one of its limitations. Without sample preparation or pretreatment, LAESI enables to detect a wide variety of classes of compounds over (from low molecular weight metabolites and lipids to proteins up to 66 kDa) down to the low-fmol level, over a dynamic range of four orders of magnitude. The diameter of ablated material is typically between 200 to 400 μm and is of 40 to 50 μm in depth.⁵³

Other techniques based on the same principle, laser desorption or ablation followed by subsequent ionization by electrospray, have been developed. As an example: electrospray laser desorption/ionization (ELDI) is a hybrid combines laser desorption with post-ionization by electrospray, for direct ambient analysis of solids. Unlike LAESI which is strictly a 2.94- μm infrared laser to desorb analytes from water-containing samples, ELDI can use either UV (i.e. N_2 laser at 337 nm) or IR (i.e. 2.7–3.1 μm) lasers, but they share a similar ionization mechanism. The laser was focused to obtain a beam with a spot size of approximately 100 $\mu\text{m} \times 150 \mu\text{m}$. To date, ELDI has mainly been employed for characterizing proteins and peptides⁵⁶⁻⁵⁷ but the applicability to the analysis of drugs of abuse only relies on a proof of concept on the illicit drug methaqualone.⁵⁸ The literature does not report any study on tissue sections so far.

2.5.2. Atmospheric pressure femtosecond laser desorption ionization (AP fs-LDI) and laser electrospray mass spectrometry (LEMS)

Atmospheric pressure femtosecond laser mass spectrometry (AP fs-LDI MS),⁵⁹ referred as laser electrospray mass spectrometry (LEMS)⁶⁰⁻⁶¹ when combined with ESI, has been introduced in 2010. AP fs-LDI uses a focused NIR femtosecond laser pulses, without the addition of any laser absorbing matrix, either native or external. The NIR femtosecond laser pulses employed have very high peak power densities, and thus allow for direct non-

resonant desorption and ionization of analytes.⁶² This explains why no matrix is required to assist in desorption and ionization. As a result, the sample absorbs the energy directly but no intact molecular ion of small molecules above 400 Da have been revealed by this technique so far. In the LEMS configuration, the femtosecond laser vaporization (laser pulses of ca. 50 fs, 1 kHz) is combined with electrospray ionization, of which charged droplets captured the ablated analytes (including non-volatile analytes) and carry them to the mass analyzer.

LEMS provides so far the highest lateral resolution of 10 μm together with LA-ICP-MS (spatial Rs = 10 μm) or LDI-MS (spatial Rs = 10 μm ⁶³), over what have been previously reported in ambient imaging analysis such as DESI or LEASI (20 – 50 μm).¹¹ The limits of detections are of the order of the pmol level for fs-LDI⁵⁹ and the fmol level for LEMS.⁶⁰⁻⁶¹ LEMS has been used on a variety of analytes such as pharmaceuticals, lipids,⁶⁰⁻⁶¹ explosives,⁶⁴⁻⁶⁵ and plant tissue organ type and phenotype.⁶⁶⁻⁶⁷

2.5.3. Desorption atmospheric pressure photoionization (DAPPI)

Desorption atmospheric pressure photoionization (DAPPI) has been introduced as a rapid tool for the analysis of compounds of various polarities on diverse surfaces.⁶⁸⁻⁶⁹ DAPPI uses a microchip nebulizer that emits a heated mix of atomized gas and solvent vapor and a UV-lamp (i.e. emitting 10 eV-photons), together directed toward the sample. The generation of ions in the positive mode occurs via photoionization of desorbed neutral analytes, charge transfer with charged solvent species or dopant molecules, and/or ion-molecule reactions involving protonated solvent/dopants.⁶⁹ The same reactions occurs in the negative ion mode ionization but with the additional electron transfer mechanisms.¹⁰ The limits of detection obtained with DAPPI were in the range of 50-670 fmol.⁶⁸

2.6. Plasma-based techniques

Since Horning *et al.* described for the first time APCI using a corona discharge at atmospheric pressure for small molecules analysis,⁷⁰ lately designed to increase the population of reagent ions by using an atmospheric pressure glow discharge (APGD),⁷¹⁻⁷² plasma-based techniques have been extensively developed and their applications have been extended to the direct analysis of surfaces. These techniques offer the advantages of requiring a simple instrumentation, minimum need for high purity solvents and generation of simpler to interpret mass spectra. Plasma-based techniques are mainly applied to low molecular weight analytes up to 800-1'000 Da depending on the technique used for desorption. They have also been attractive for miniaturization to open possibilities for ambient MS field applications.⁷³⁻⁷⁴

2.6.1. Direct analysis in real-time (DART)

Among the emerging plasma-based techniques, the so-called direct analysis in real-time (DART) is the most popular. DART was developed almost at the same time as DESI by Cody and co-workers.⁷⁵ DART relies upon the fundamental principles of APCI as it uses a nitrogen

or helium glow discharge to ionize molecules from the sample surface. The gas is introduced into a discharge chamber containing a cathode and an anode and an electrical potential of several kV initiates an electrical discharge producing ions, electrons, and excited-state species in a plasma. The gas flows into a second chamber where a second perforated electrode can be biased to remove ions from the gas stream. The gas flow then passes through a third region that can be optionally heated.⁷⁵ The DART gas flow can be directed i) directly toward the mass spectrometer orifice (i.e. in this case the sample is held in-between the gas flow and the entrance of the mass analyzer as shown Figure II- 1), or ii) reflected off the sample surface and then the desorbed/ionized molecules enter into the mass spectrometer.

The formation of ions in DART relies on multiple parallel pathways such as i) ionization occurring by collisions with electronically excited metastable helium atoms; or ii) proton transfer to the analyte from hydronium ion water clusters, depending on the water vapor pressure and ambient temperature in the open air ionization region. The prevalence of one mechanism over the other is strongly dependent on the analyte physico-chemical properties.¹⁰

DART is described as a very versatile technique for sampling hundreds of polar and nonpolar chemicals (e.g. chemical warfare agents and their signatures, pharmaceuticals, metabolites, peptides and oligosaccharides, synthetic organics and organometallics, drugs of abuse, explosives, and toxic industrial chemicals) on various surfaces (e.g. asphalt, human skin, currency, airline boarding passes, business cards, fruits, vegetables, spices, beverages, body fluids, plastics, horticultural leaves, cocktail glasses, and clothing).

2.6.2. Plasma-assisted desorption ionization (PADI)

PADI is a recent development by McCoustra and co-workers⁷⁶⁻⁷⁷ using a cool plasma source to ionize and desorb molecules from a surface. So far, PADI have only been used for few applications but has shown promising results for forensics applications including direct analysis of explosives, direct analysis of cocaine from human skin, determination of active ingredients directly in drug tablets, and analysis of toxic and therapeutic compounds in complex biological samples.⁷⁷

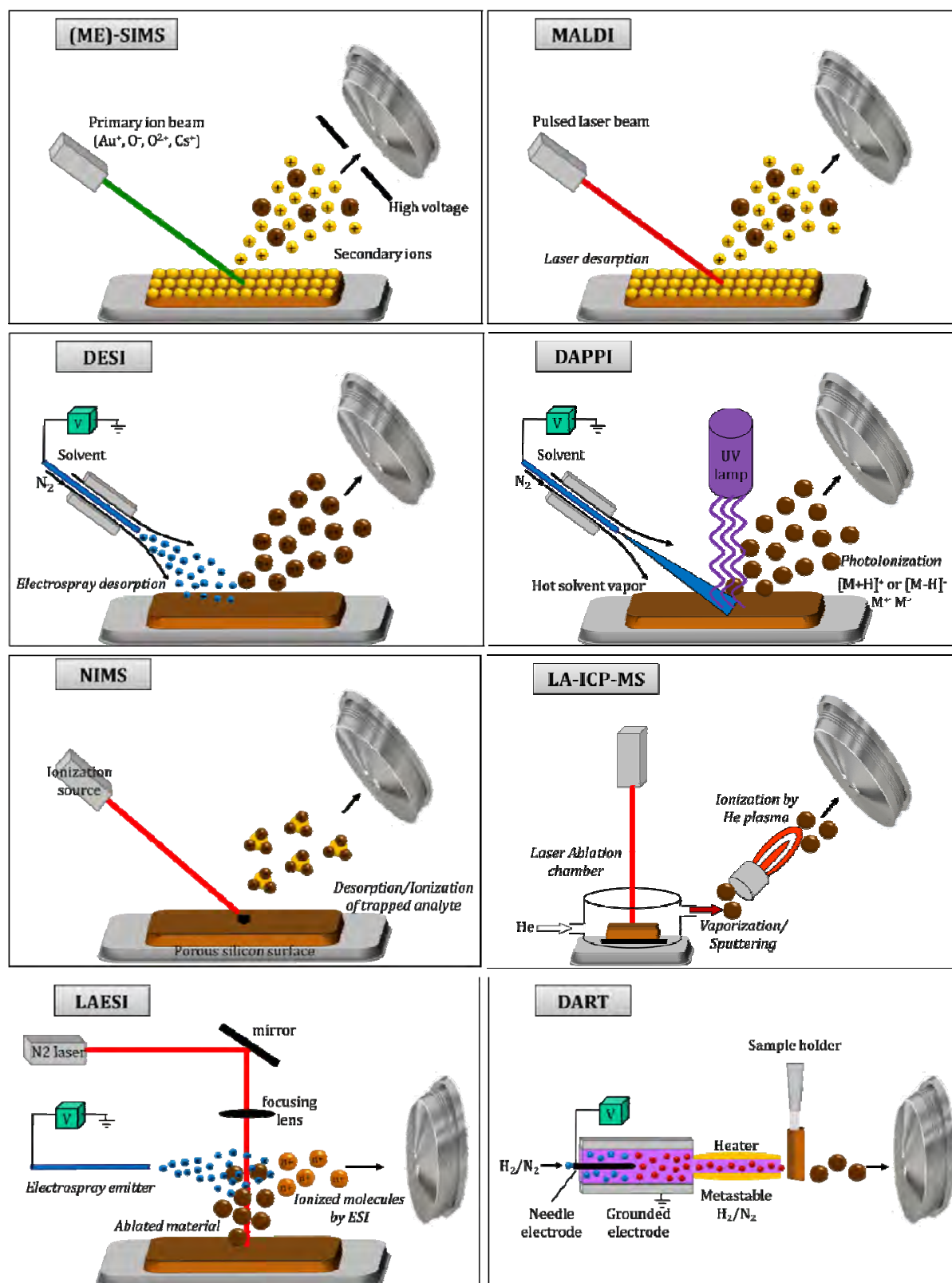


Figure II- 1. Schematic representations of some of the desorption/ionization techniques used in forensic toxicology and plant biology.

2.7. Applications of MS imaging

2.7.1. Application of MSI in forensic toxicology

a) Single hair samples

Body fluids or hair can reveal recent drug of abuse intake, but only hair provides information about chronic consumption. Current hair analysis is mainly based on gas or liquid chromatography coupled to MS(/MS). It can reveal the user's drug intake history but requires multiple sample preparation steps beforehand (*e.g.* washing, pulverization, overnight hair digestion, extraction, and derivatization). Additionally each hair sample has to be cut into several 1 cm-long segments, which are pulverized and analyzed individually to reconstitute the drug consumption from scalp to hair tip (*i.e.* from present to past).

Recent studies have proposed to image the distribution of illicit drugs in single hair samples by MALDI-MS(/MS), either longitudinally sectioned⁷⁸⁻⁷⁹ or as intact hair.⁸⁰ For longitudinal section analysis,⁷⁸ the time required for imaging in pixel mode the hair sample was about 2 hours with a high resolution, high laser frequency TOF/TOF instrument. In the intact hair study, we described how to generate an image of the cocaine distribution over several months (*i.e.* using a hair sample of ca. 10 cm) in less than 10 minutes. This was achieved with a QqQ_{LIT} instrument operated in SRM mode combining a 1'000 Hz-frequency laser with a relatively high MALDI plate speed (*i.e.* rastering performed every 1 mm at 1 mm/s, Figure II- 2.A).⁸⁰ Qualitative and quantitative data were acquired to confirm the cocaine intake: i) by using targeted MS/MS experiments to confirm the drug identity (Figure II- 2.B), and ii) with a relative quantification from the scalp to the tip and a simultaneous determination of the benzoylecgonine (*i.e.* a cocaine metabolite) to cocaine ratio (BZE/COC), which must be greater than 0.05 to prevent false positive results due to external contamination (Figure II- 2.C). The method allowed monitoring the consumption from chronic users with a sensitivity of 5 ng/mg, which covers a wide range of real cases, typical cocaine concentrations in hair range from 0.5 (cut off from the Society Of Hair Testing) to over 200 ng/mg.⁸¹⁻⁸² Similarly, the direct MALDI-TOF analysis of single intact hair (4-cm segments) containing 100 ng/mg of cocaine was investigated,⁸³ but did not provide MS imaging results due to a lack of sensitivity.

In addition to MALDI, DESI was used for a rapid screening of anabolic steroid esters in bovine incurred hair for hormone and veterinary drug analysis and forensics.⁸⁴ However, the inefficient desorption ionization and the low quantities of steroid esters present in the surface sampled did not give any satisfactory results and necessitated a simplified ultrasonic liquid extraction. TOF-SIMS has also been successfully applied to provide molecular distribution of external chemicals such as hair dye ingredients in longitudinally sectioned single hair⁷⁹ and to image arsenic traces in human hair cross-sections.⁸⁵

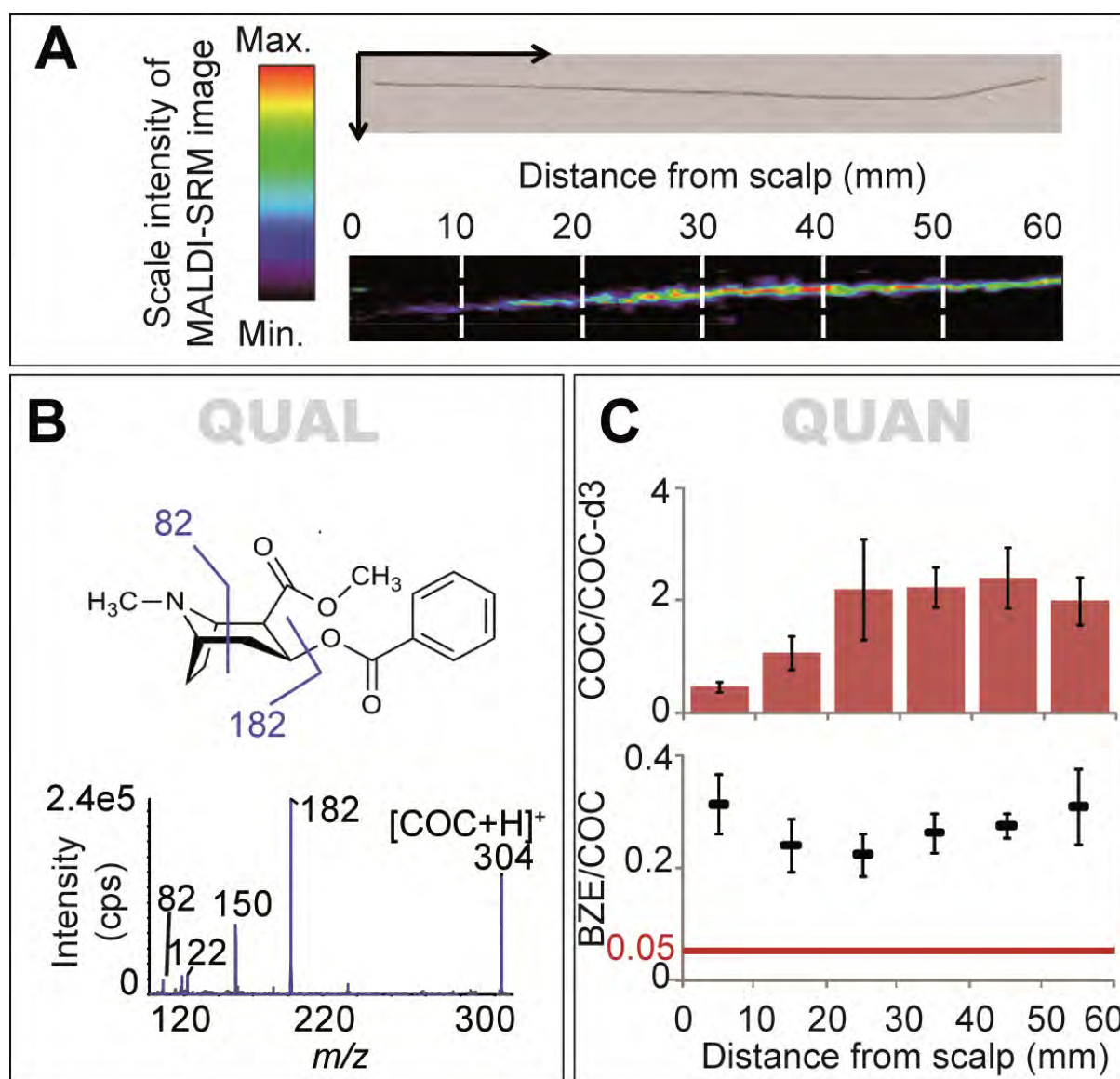


Figure II- 2. (A) MALDI image of cocaine distribution in a single hair sample based on the SRM trace of COC (m/z 304 > m/z 182); (B) structure and MS/MS spectrum (precursor ion m/z 304) from hair sample with specific fragment ions (m/z 82, m/z 122, m/z 150, m/z 182) confirming the identity of COC; (C) histogram showing the distribution of COC in hair sample from scalp to tip and BZE/COC ratio ($n=5$). QUAL: qualitative; QUAN: quantitative. Adapted from Porta *et al.*⁸⁰

b) Tissue samples

Detection and quantification of drugs in tissue usually require homogenization, extraction and/or derivatization of the analytes of interest, and other optional steps to remove lipids or other interfering endogenous compounds prior to the MS analysis. As a consequence, the analyte spatial distribution is lost. For MS imaging, sample preparation for direct analysis of postmortem tissue only consists of a few steps: i) organ/tissue deep freezing, ii) cut 10-20 μm -thickness slices, iii) transfer the tissue slices onto a sample holder (*e.g.* glass slide, stainless steel plate), and iv) apply the matrix on the top when MALDI-MSI analyses are performed. This rapid protocol allowed the quantification of cocaine in autopsied brain or kidney of human cocaine or morphine users.⁸⁶⁻⁸⁹

c) Latent fingerprinting

Finally, another area where MS imaging techniques are emerging in forensics is the reconstitution of latent fingerprints (LFP). LFP can help to identify an individual and give potential evidence of prior contact with explosives or illicit substances to prove his/her connection with a crime scene. LFP can be reconstituted from different surfaces (*e.g.* glass, paper, plastic) without any sample pre-treatment under its native environment by DESI,⁹⁰ SIMS,⁹¹⁻⁹² GALDI⁹³ or even by applying a MALDI matrix.⁹⁴⁻⁹⁷ Figure II- 3. is an example of the potential of MALDI to study latent finger marks of endogenous lipids (*e.g.* oleic acid, stearic acid, cholesterol).⁹⁴ The technique distinguishes between overlapping fingerprints belonging to different individuals, relying on the fact that they present distinct exposure to chemicals.^{90, 98} Another advantage of this technique is that the resolution achieved is sufficient to clearly distinguish the ridges and the minutiae that can be exported into fingerprint recognition software.⁹⁹ The ability of SIMS to detect exogenous contaminants (*e.g.* amphetamine drugs, gunpowder residues, arsenic, cosmetics)^{91-92, 100} has also been demonstrated. Using gold nanoparticles as a fingerprint development powder, fingerprints can be optically visualized and molecular images of fingerprints obtained *via* SALDI-imaging.⁹³

The proof of concept of *in vivo* analysis of human fingertips have also been performed on volunteers by DESI.¹⁰¹ In combination with miniaturized mass analyzers, this led us expect to perform real-time analysis directly on the crime scene, at the time of the first investigations.

d) Investigation of counterfeit pharmaceuticals and illicit drugs in drugs tablets and powders

The investigation the chemical composition of drug tablets and powders may be of utmost relevance to prove of counterfeit pharmaceuticals. As a consequence, the development of rapid detection and identification methods are mandatory. There is also another case where such fast approaches are required to save precious time for diagnostics: in emergency departments of hospitals for instance, where patients arrive with unknown tablets in their pockets. Conventional methods consist in dissolving the tablets or the powders in an appropriate solvent, followed by filtration and/or centrifugation step(s). The recovered solution is then subsequently analyzed by liquid chromatography (LC) coupled to a UV diode array detector (UV-DAD) or with gas chromatography coupled to mass spectrometry (GC/MS).¹⁰² Direct analysis offer the possibility of performing such rapid analysis, and again, with the use of miniaturized mass analyzers, real-time analysis can be envisaged. The techniques that have been used are mainly DESI¹⁰²⁻¹⁰³ and DAPPI, the latter has been proven particularly suitable for the direct analysis of a wide variety of drugs of abuse investigated of confiscated drug samples of various forms such as tablets and powders, herbal products, and anabolic steroids in oil and tablets.^{68, 104-106} A method based on SALDI (*i.e.* DIOS) for MDMA impurities profiling has been developed to trace the origin of MDMA pills.¹⁰⁷

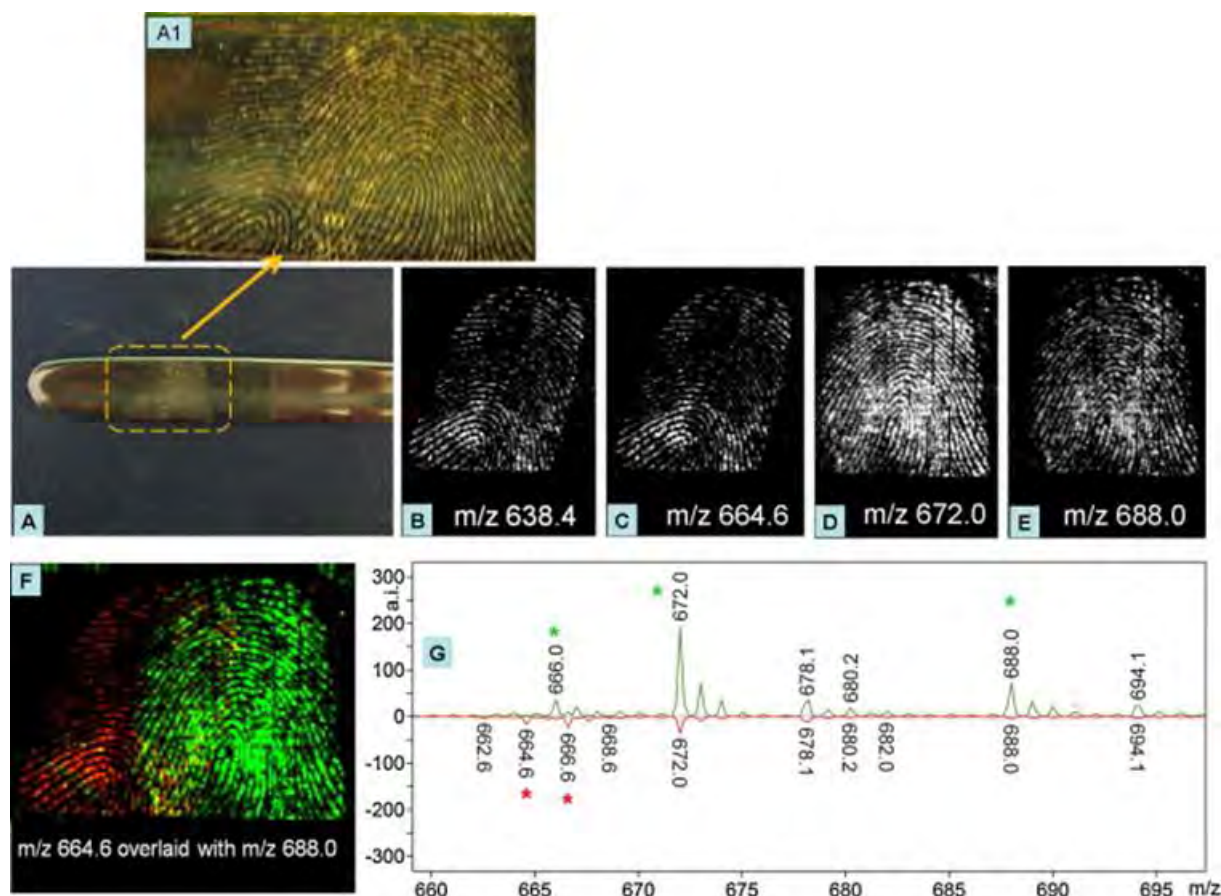


Figure II- 3. MALDI MSI separation of overlapping ungroomed fingermarks deposited on a kitchen knife by two different donors. Panel A shows the optical image of the dusted overlapping fingermarks and inset A1 shows a zoom in of the overlaid marks. Two ion images per fingermark are reported to demonstrate separation (B–E). Panel F shows the image of the ion signal at m/z 664.6 overlaid with the image of the ion signal at m/z 688.0 which are characteristically present in the mass spectrum profiles of the two impressions (G). The vertical lines visible in D and, to a lesser extent in E, are due to how the tape was handled in the process of lifting. Adapted from ref.⁹⁸

e) Trace detection of explosives

Although it does not involve the concept of imaging but still relies on the simple and direct analysis of samples under ambient conditions, this section reports few applications regarding the direct, trace level detection of explosives on ambient surfaces. There is currently an urgent need for the development of a rapid technique to detect explosives. This is actually a subject of continuing strong interest in analytical chemistry, driven by threats to civil society and by environmental problems associated with explosives residues. The ideal technique should be highly sensitive, applicable to non-volatile and thermally unstable analytes, highly specific response times and should not require any sample preparation or sophisticated handling. This has pushed forward the development of techniques such as DESI.¹⁰⁸ Takàts and co-workers have shown the analysis of within 5 s, and the benefit of adding reactants in the solvent system to increase the selectivity by the production of ions as the chloride and trifluoroacetate adducts the well know explosive 2,4,6-trinitrotoluene TNT.¹⁰⁹ Laser electrospray mass spectrometry (LEMS) has also been employed for the detection of sub-microgram quantities of explosive samples on a metallic surface.⁶⁴⁻⁶⁵

f) Analysis of inks and document verification

The analysis of inks and verification of documents, including banknotes, printing and writing papers, are one of the fields of forensics where direct surface analysis is useful for rapid investigation. It has been done either by DESI,^{47, 110} EASI,⁴⁷ DART¹¹¹⁻¹¹² or ToF-SIMS (imaging).¹¹³ Particularly, LDI has been used for investigating colorants and dyes found in inks¹¹⁴⁻¹¹⁷ and for characterizing ink degradation over time and after exposure to light, as a function of the type of paper and ink formulation.¹¹⁸⁻¹²⁰ The LDI analysis of the pigments present on old documents also provides proof of their authenticity.¹²¹⁻¹²²

g) Biological fluids

The ability to detect illicit drugs directly in oral fluids is of major interest for roadside, workplace and athlete drug testing. Some publications report the extension of the application of direct surface analysis to biological fluids such as plasma, blood, urine or saliva by DAPPI, DESI, NIMS or SALDI/DIOS. All of these techniques were hyphenated to mass spectrometric detection for the rapid screening of xenobiotics such as drugs of abuse.^{29, 123-125} These techniques are reported to perform sensitive, simple, and rapid analyses from highly complex biological tissues and fluids, with minimal to no sample preparation beforehand. Matrix effect in the analysis of drugs of abuse from urine has been evaluated between DAPPI-MS and DESI-MS and the results have indicated better matrix tolerance of DAPPI over DESI. Nonetheless, compared to common GC-MS/MS analysis, sample pretreatment was still required to attain equal sensitivities.¹²³

h) Cosmetics

Trace of cosmetics can constitute reliable evidence in a crime scene, the application of direct surface analysis in the area of cosmetics may provide useful tool for toxicological and forensic analysis to obtain accurate and reliable results in a rather quick and straightforward way. Recently, de Oliveira *et al.* described an easy and rapid technique to evaluate lipids and find compounds of interest that differentiate the samples in lipsticks using sorptive tape-like extraction coupled with LDI-MS imaging (STE-LDI-MSI). No sample preparation and no matrix are required in this novel cosmetic assessment tool.¹²⁶ Cosmetic analysis using MALDI-MSI has also been demonstrated on products of daily use, namely nail polish, lipsticks and eyeliners of multiple brands sold in the worldwide market.¹²⁷

If nanoSIMS has been investigated as an imaging technique to detect cosmetic ingredients in the skin, it cannot be operated in and does not stand for a potential tool to be used in the case of forensic investigation for such samples (except hair samples as previously described).¹²⁸

2.7.2. Application of MSI in plant biology

With the increasing success of MS imaging technologies in biomedical sciences for biomarker discovery and drug localization, MS imaging has rapidly become attractive to biologists as the solution to answer biologically relevant questions. Like for forensics samples, the majority of MS imaging experiments involve snap-frozen, chemically unmodified material, embedded or not. Plant material includes leaf, stem, fruit, flower, grain. Like for animal tissue sectioning, the sample preparation remains challenging. And because of the inherent diversity in the composition of plant tissues, the optimization of the sample preparation is sample dependent.¹²⁹ If some samples remain difficult to section (i.e. leaves, pollen and grains), some alternative sample preparation method can be used, such as the use of adhesive tape to fix it on the target plate, in order to study intact leaves for instance (see ref.¹³⁰ and Chapter 3, Part 3). Appropriate methodology has also to be applied in function of the objective of the study (e.g. analysis of intact sample or cross sections).

a) Localization of accessible and highly abundant metabolites in various plant materials

Initial studies have reported the ability of the MSI approach to localize and determine the relative abundance of metabolites, easily accessible and/or highly abundant (e.g. the cuticle layer of plants), at relatively high spatial resolution (i.e. 100 μm). To illustrate, MALDI-MSI has been used to highlight the uneven distribution of specific amino acids, sugars and phosphorylated metabolites in wheat seeds at various development stages and under temperature stress.¹³¹ This work presents results from cryosections, but in some case where it's difficult to obtain good sections of material, it's possible to blot the freshly cut surface of plant material directly on precoated cellulose membranes, as it was used with potato tuber material. The distribution and identification of a phase I metabolite after exposure to pesticides has also been highlighted by MALDI-MSI in both the soybean stem¹³² and the whole plant of sunflower.¹³³ The identity of several glucosinolates, that are multi-functional plant secondary metabolites which play a vital role in plant defense, have also been confirmed by MSI and their distribution imaged in *Arabidopsis* leaves¹³⁴ and flowers.¹³⁵ MALDI-MSI allows for investigating the distribution of various metabolites such as carbohydrates (e.g. cellulose in poplar stem,¹³⁶ fructans in onion bulbs¹³⁷), phospholipids in rice seeds,¹³⁸ γ -aminobutyric acid in eggplant,¹³⁹ carbohydrates in wheat stem¹⁴⁰ and complex secondary metabolites such as diterpene glycosides.¹⁴¹ Others studies report the imaging of surface lipids and metabolites that compose the cuticular waxes on intact plant tissue by MALDI-MSI,¹⁴²⁻¹⁴³ LDI-MSI,¹⁸ GALDI-MSI^{21, 144} or LDI-MS using silver colloid as matrix.^{22, 142}

Alternative approaches to common UV-MALDI-MSI consist in using IR-MALDI-MSI, which uses the native water within the tissue as natural matrix.¹⁴⁵ The rapid metabolic profiling of *Nicotiana tabacum* defence responses against insect attack has been performed by IR-LDI-MSI.¹⁴⁶ LAESI-MSI, which also uses a focused mid-IR laser and water-content of the tissue,

has proven suitable for imaging metabolites in plants, particularly for depth profiling capability for 3D imaging.¹⁴⁷

Due to its successful applications for the direct characterization of alkaloids,¹⁴⁸ glycosides¹⁴¹ and Salvorin A¹⁴⁹ in plant tissues and previously applied to the imaging of natural products in algal tissue,¹⁵⁰⁻¹⁵¹ DESI-MS has been identified as a promising candidate for imaging plant material. DESI has enabled the direct identification of Chlorophyll catabolites in senescent leaves from three higher plants (*C. japonicum*, *L. styraciflua* and *O. virginiana*), after removal of the waxy layer with chloroform.¹⁵² This paper also reports a strategy involving the production of leaf imprints, generated from freshly collected leaf samples placed on the top of polytetrafluoroethylene (PTFE) material. Because preliminary direct DESI analysis of leaf showed that despite surface pretreatment, Chlorophyll catabolite related ions were abundant only at certain hotspots on the leaf surface, due to possible epidermal cells and residual waxes which constitute a mechanical barrier to extraction by charged microdroplets. The imprinting technique using PTFE as substrate for imprints enables to overcome that issue, since it offers the advantage of a more efficient desorption, resulting in more intense and stable signal compared with other surfaces.¹⁵² Direct analysis of tissue imprint was found suitable for visualizing metabolite distribution and enzymatic conversion in plant tissues.¹⁵³ Laser electrospray mass spectrometry (LEMS) has recently emerged in plant biology as a potential tool for discriminating eight phenotypes from a single plant organ class and to find potential biomarkers.⁶⁷

b) Localization of metabolites in individual cells

The ability to image the location of molecules to individual cells is providing unprecedented data concerning the location of metabolites. Although not widely used in plant biology, SIMS has also been used for some interesting applications, due to ability to provide high resolution imaging down to the μm scale. Therefore, ToF-SIMS has been used to trace the transport routes of macronutrients in plants at the level of cells and tissues and to measure their elemental distributions, providing useful information about the dynamics and structure-function relationships of transport processes¹⁵⁴ and also to characterize water movement between cells in a soybean plant body, which is the basic phenomenon of plant solute transport.¹⁵⁵ ToF-SIMS allows direct mapping of morphological distribution of lignin units at the cellular level, in the xylem of maple.¹⁵⁶ Finally, flavonoids have been imaged in fresh seed sections of peas (*Pisum sativum*) and *Arabidopsis thaliana* (Figure II- 4) at a spatial resolution better than 5 μm .¹⁵⁷

Even though they do not compete with subcellular spatial resolution (R_s) achievable with SIMS, other techniques have been employed for *in situ* cell-by-cell imaging of plant tissues: LAESI (spatial R_s down to 20 μm ^{54, 158-160}); MALDI-MSI in cotton seeds (spatial R_s = < 50 μm , employing the oversampling strategy¹⁶¹); LA-ICP-MS (spatial R_s = 10 μm); GALDI (spatial R_s = 50 μm with oversampling²¹), silver-assisted LDI (spatial R_s = 12 μm ²²). Particularly, because in principle at least any UV-absorbing compound can be imaged, LDI-MSI is particularly advantageous for the detection of metabolites containing condensed aromatic

rings, such as flavonoids, flavanes, anthocyanins and other related plant pigments, as shown from a comparison of *A. thaliana* and *Hypericum* species secondary metabolites, where imaging experiments have been performed at a resolution of $R_s = 10 \mu\text{m}$.⁶³

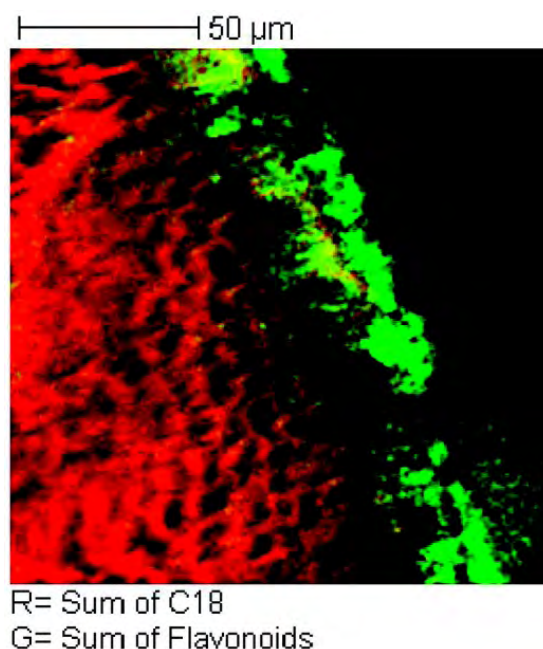


Figure II- 4. Example of a high spatial resolution ToF-SIMS image acquired in the negative ion mode. This image was recorded at the edge of an *A. thaliana* seed section embedded in a polyester resin (field of view $150 \mu\text{m} \times 150 \mu\text{m}$, 256×256 pixels, pixel size 586 nm). Two-color overlay: red, sum of C_{18} fatty acid carboxylate ions; green, sum of flavonoid ions. From Seyer *et al.*¹⁵⁷

This image clearly indicates that the coat is represented by the flavonoid ion signals, while the fatty acid ion signals are more intense in the cotyledon.

c) Genomics

The ability of MS imaging approaches to perform single cell analysis provides new perspectives in genomics. After integration of genetically induced changes in the metabolome of the tissue of interest, some papers report the ability of MSI to visualize of this unknown difference and thus to localize the gene expression by monitoring the biochemical alteration of the genetic lesion.^{21-22, 142, 162-163}

d) Proteomics

Some publications report the use of MS imaging as a direct approach to proteomic study in plant biology. Imaging LA-ICP-MS, initially applied to investigate metal distributions in plant (e.g. to study the uptake and transport of nutrient and toxic elements or environmental contamination), has been combined with proteomic studies to identify metal-containing proteins and also phosphoproteins.¹⁶⁴ Usually, the plant material proteome is previously resolved by 2D separation techniques, followed by a direct ionization hyphenated to mass spectrometry detection. MALDI-TOF(/TOF) has also been used in different studies in combination with 2D separation techniques. This has enabled for instance to evaluate the early cytokinin response (i.e. cytokinins are plant hormones involved in regulation of diverse developmental and physiological processes in plants such as infection) through the identification of differentially expressed proteins and phosphoproteins of *A. thaliana*.¹⁶⁵⁻¹⁶⁶ MALDI-TOF(/TOF) also enabled the evaluation of metal-ion stress in sunflower leaves,¹⁶⁷ the comparative analysis of proteomic changes in contrasting flax cultivars upon cadmium exposure,¹⁶⁸ the characterization of the adaptive response of Egyptian barley land races to drought stress through proteomic changes.¹⁶⁹

e) *In vivo* analysis of plants

Recent developments have contributed to the *in vivo* analysis of plants and characterization of plant materials, to provide real-time information on sugars, amino acids, fatty acids, lipids, and alkaloids. The principle of the “leaf spray” is very simple: an electrical potential is applied to the plant and its natural sap, or an applied solvent, generates an electrospray that carries endogenous chemicals into an adjacent benchtop or miniature mass spectrometer (Figure II- 5.A,B). The sharp tip needed to create a high electric field can be either natural (e.g. bean sprout) or a small incision can be cut in a leaf, fruit, bark, etc. Stress-induced changes in glucosinolates can be followed on the minute time scale in several plants, including potted vegetables. Differences in spatial distributions and the possibility of studying plant metabolism are demonstrated.¹⁷⁰

An alternative approach, “*in vivo* nanoelectrospray” uses a capillary partially inserted into the plant to sample liquid from a highly located region, and then, a high voltage is applied to the plant to generate an electrospray from the capillary tip for MS analysis (Figure II- 5.C). Using this method, bioactive molecules such as saccharides, glycoalkaloids, flavonoids, organic acids, and glucosinolates (GLs) are detected in the target regions of living plants or fresh fruits.¹⁷¹ Using a nanoelectrospray tip, it is possible to extract the contents of a single leaf, stem, or petal cell from a plant to extract specific metabolites.¹⁷²

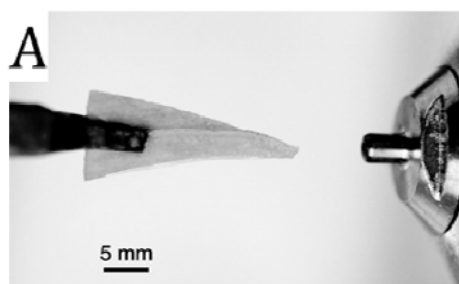
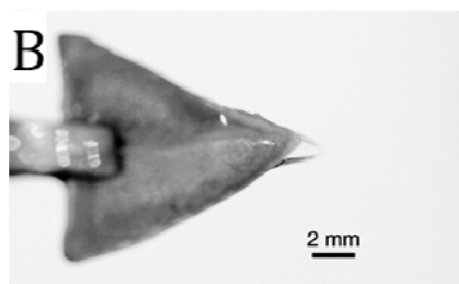
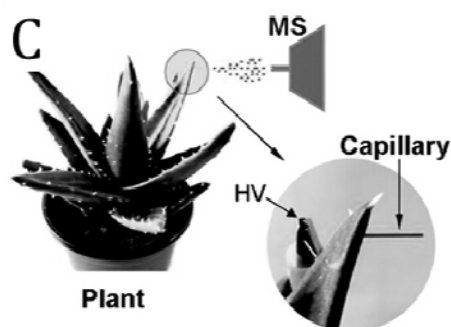


Figure II- 5. (A) Photograph of leaf spray ionization of green onion leaf cut to a point and held by a high voltage connector in front of the atmospheric inlet of a mass spectrometer. From ref.¹⁷⁰



(B) Photograph of leaf spray ionization of spinach leaf in negative ion mode. The spinach leaf was cut into a triangle, and methanol was applied on the leaf to achieve leaf spray ionization. From ref.¹⁷⁰



(C) Schematic diagram of *in vivo* nanoelectrospray. From ref.¹⁷¹

2.8. Summary and perspectives

Although MALDI is still the main technique used in MS imaging, and in direct analysis in general (i.e. not relying on imaging), MALDI has to face a growing number of promising techniques operated under ambient conditions. These techniques allow for rapid analysis of untreated samples (i.e. kept in their native state), sometimes in real-time, and even performed *in vivo*, as it has been shown for the direct detection of drugs of abuse from human skin or for analyzing bioactive molecules in plant. Selective and sensitive direct approaches are presented as a very powerful tool for forensic investigations and in plant sciences, and provide numerous advantages that can be exploited by both fields.

The sample preparation prior to MS analyses is considerably reduced enabling an enhanced analytical throughput, which is not negligible in forensics. The most of the techniques used in the field of forensics allow for a very few consumption of the material the spatial distribution of the drugs and their metabolites is kept intact within the samples, which can reveal decisive information in the case of latent fingerprints or hair analysis to monitor cocaine consumption for example. In the case of hair analysis for instance, and since only part of the strand is analyzed, additional analyses are possible at any time on the remaining hair from the strand which is of importance in the field of forensic investigations. Moving the ionization process from vacuum into the atmospheric environment was undoubtedly a development breakthrough in mass spectrometry (MS). The development of ambient mass spectrometry is still growing with the emergence of techniques and has been pushed in forensics by advances in MS miniaturization, which improve the prospects for on-scene chemical identification, reducing sample turnaround times.¹⁷³ In forensics, a spatial resolution of several hundred micrometers is sufficient to generate relevant data. However, direct ambient analysis can suffers from the lack of a chromatographic step prior to MS detection which is particularly critical for isobaric or phase II metabolites (in case of in-source fragmentation). In that respect, differential ion mobility spectrometry (DMS) offers an interesting alternative to chromatographic separation because it enables the separation of isobaric analytes and was recently successfully applied in combination with liquid extraction surface analysis (LESA) for the analysis of drugs in human tissues.⁸⁸

Advanced developments in MS imaging approaches (see Chapter 1, Part 2) allowed for the identification of metabolites from various plant materials. So far, few proteomic studies based on MS imaging have been carried out in plant tissue and applying (MALDI-)MSI in plant biology will probably be the next challenge in the field. This would allow for providing precious information about the spatial co-localization of metabolites and proteins and thus a better understanding of the elucidation of metabolic networks and their regulation.

At the frontier between plant biology and forensic investigations, it is clear that MALDI, SIMS and ambient ionization techniques have a role to play in food-quality and safety analysis,¹⁷⁴⁻¹⁷⁵ which is gaining of interest for developing new analytical techniques.

Particularly, the determination of contaminants such as fungicides in food items is an ongoing trend in applications due to the rapid screening abilities of the ambient techniques in particular, such as DESI¹⁷⁴ or EASI, which has been demonstrated suitable for metabolomics and quality control of vegetable and animal.¹⁷⁶⁻¹⁷⁷ Extraction electrospray ionization has been also used for the differentiation of maturity and quality of fruits and for the rapid fingerprinting and classification of extra virgin olive oil.^{48,178} Recent publication has also introduced a platform based on liquid extraction surface analysis (LESA) mass spectrometry as an interesting platform for the qualitative determination of surface chemical residues resulting from the artificial spraying of selected fresh fruits and vegetables with representative pesticides.¹⁷⁹ LESA could also be an interesting platform to be used in plant biology and have a great potential for forensic toxicology (see Chapter 4 of this thesis manuscript).

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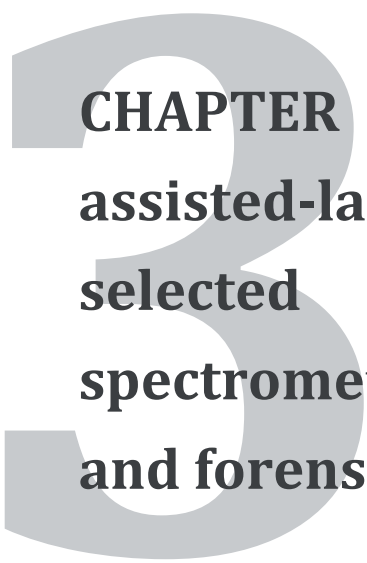
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CHAPTER 3. Application of matrix assisted-laser desorption/ ionization selected reaction monitoring mass spectrometry imaging to plant biology and forensics

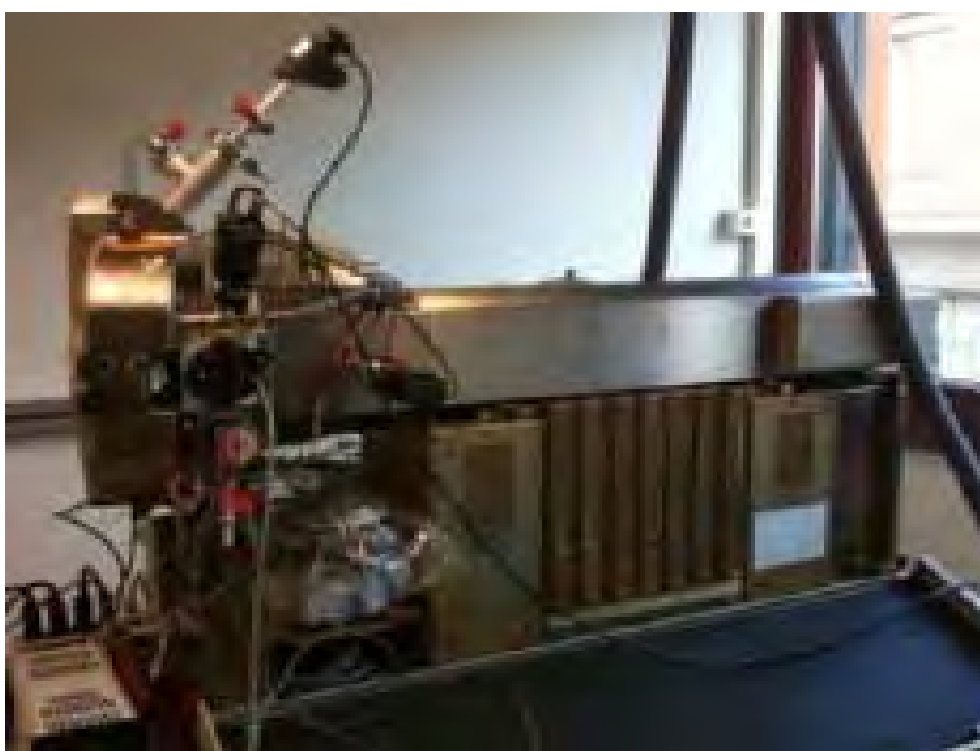
This third chapter presents the applications of the analytical platform introduced by Hopfgartner *et al.*¹ and Prideaux *et al.*² based on matrix-assisted laser desorption/ionization hyphenated with selected reaction monitoring mass spectrometric (MALDI-SRM/MS) detection. The objective of these studies is to develop and apply this platform for targeted analysis and imaging of low molecular weight compounds in plant biology and forensic toxicology.

Although the application of MALDI-MSI constitutes the main core of this chapter, other surface sampling and ionization techniques operated both under ultra-high vacuum (i.e. secondary ion mass spectrometry) and ambient pressure (i.e. liquid extraction surface analysis) are presented as complementary tools to MALDI to study drugs and their metabolites from biological samples, i.e. plant leaves, postmortem human tissues and hair.

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 1 – Mass spectrometry imaging on a MALDI-QqQ_{LIT} platform



3.1.1. Principles of ion formation in matrix-assisted laser desorption/ionization (MALDI)

Matrix-assisted laser desorption/ionization (MALDI), invented in the 1980s by F. Hillenkamp and M. Karas,³⁻⁵ is a widespread analytical method and the most commonly used ionization technique in the MS imaging field to date. MALDI is often viewed by users as *a mixture between an analyte and an excess of matrix material, that usually co-crystallized from solution and is later irradiated with a laser of which energy is absorbed by the UV-absorbing matrix of which role is to promote the analyte ionization by proton transfer reactions*. However, the underlying mechanisms leading to the formation of matrix and analyte ions are actually more complex. It comprises the formation of protonated, deprotonated, cationized and radical species, relatively independent of the matrix, the solvent composition, the solution pH and even analyte acid-base properties.⁶ The ion formation mechanisms in UV-MALDI fundamentally rely on two steps: the first step involves primary ionization occurring during or shortly after the laser pulse, followed by secondary reactions in the expanding plume of the desorbed material.⁷

This section reviews these principles and also describes some of the parameters that may be critical for MALDI-MS imaging experiments.

3.1.1.1. Laser desorption and ablation phenomena

In its initial form, **laser desorption ionization** (LDI), i.e. MALDI without the need for a UV-absorbing matrix, was performed on non-volatile analytes of up to 1-2 kDa, on the condition that the analytes possess at least one UV-absorbing chromophore moiety. Even if LDI has some advantages over MALDI, especially in the imaging field where no matrix is required (i.e. it prevents the potential delocalization of analyte during the application of a wet matrix on the top of the sample), experiments have revealed the presence of highly abundant species, such as endogenous metabolite species in plants⁸⁻⁹ or endogenous lipids in mouse brain tissues¹⁰⁻¹¹ (data related to LDI-MSI experiments). Together with its overall low sensitivity, the need to analyze larger (bio)molecules led to the development of MALDI, which consists of i) mixing the analyte with a matrix in excess in “regular” MALDI-MS experiments or ii) coating the surface of the sample in MALDI-MS imaging experiments.

To be efficient, the **matrix**, usually a small organic compound, should possess some key properties and provide essential functions such as:¹²⁻¹³ i) strong absorbance at the laser wavelength used (typically, 337 nm with N₂ laser or 355 nm with Nd:YAG laser in UV-MALDI) to transfer the energy from the laser beam to the sample; ii) ability to dilute and isolate analyte molecules from a solid solution upon evaporation of the solvent; iii) during the disintegration of the condensed phase, the release of the embedded analyte molecules should be done without excessive destructive heating (i.e. the local temperature of the sample can reach 600 to 1'200 K¹⁴⁻¹⁵); iv) production of thin, homogeneously distributed crystals over the surface of the sample after crystallization (particularly true for MSI) and v) promotion of the ionization of the analyte.

The disintegration of the sample, after irradiation with the laser, may be characterized as “desorption” or “ablation”, depending on the laser fluence (i.e. energy per laser pulse and unit area):¹⁵

i) at low laser fluences, the transition from solid to gas phase is smooth at the top surface of the sample, resulting in the **desorption** of material containing little or no condensed particles, droplets or clusters;

ii) at higher fluences, **ablation** of the sample occurs, due to sufficient overheating, which leads to a “phase explosion” which provokes the nucleation of subsurface material. The resulting materials ejected form a dense “plume” or “gas jet” of matrix neutrals which entrains analyte molecules. This plume also contains reactive species, such as matrix radicals, electrons, and hydrogen atoms, which expand into the vacuum of the mass spectrometer ion source.¹⁶ Also, this plume can contain non-vaporized aggregated materials that are not available for MS analysis. If the laser pulse is sufficiently short compared to thermal and mechanical relaxation of the irradiated volume, the material can experience stress confinement and this leads to the ejection of larger layers of material.¹⁷

3.1.1.2. Ion formation in UV-MALDI (1): two models to describe the primary ionization mechanisms

The processes governing the generation of primary ions in MALDI are still debated by experts in the field, and several models have been proposed.^{7, 18} One of the oldest assumes that neutral analyte molecules are incorporated in the matrix crystals and the initial step for the generation of ion is a photoionization of the matrix molecules,^{4, 19} then followed by charge transfer to the analyte molecules in the plume. But this model has been supplanted principally by two modern models that will be reviewed herein: the cluster model (i.e. the lucky survivor model) and the photoexcitation/pooling model.

a) The cluster model

The mechanisms proposed to explain the major processes involved in the generation of charged analytes according to the cluster model are summarized Figure III- 1.

The Lucky Survivor model, proposed and largely described by Karas *et al.*, is one of the best explanations of the cluster model and relies mainly on photoionization and photochemical reactions. The Lucky Survivor model assumes the ions are preformed in the preparation solution and that they retain their charge in the solid matrix, which means that they are incorporated into the host matrix as charged species. Following this incorporation, the formation of singly and multiply charged initial clusters (i.e. matrix, analytes and ionic species embedded in the matrix crystals) is due to the desorption/ablation and constitute a significant portion of the ejected plume.²⁰ This is because an extensive and rapid disintegration of the solid matrix may lead to an excess of positive or negative charge in the plume. From a statistical point of view, the overall ionization yield will be functioning with a deficit/excess of anions or cations.⁶

Most of the preformed ions become re-neutralized extensively in the plume within desorbed clusters of matrix and analyte.^{6, 21} These neutralizations often occur by electrons, or matrix anions¹⁶ in the case of positive ions.⁷ As an example, a protein prepared in a solution at pH=2 will contain a high number of excess positive charge that will be compensated by counter anions (from the acid TFA for instance, the matrix anions or contaminants such as Cl⁻).

In the case of multiply charged ions, two phenomena can be observed: 1) soft desolvation resulting in the loss of neutral matrix molecule; 2) hard desolvation, resulting in the ejection of charged matrix molecules (Figure III- 1).²²⁻²³ Simple cluster evaporation may free the ion if the analyte is already singly charged.

The photoionization of matrix clusters also plays an essential role in charge generation and separation. But while at least two or three photons are required for the direct photoionization of individual gas phase matrix neutrals, the pooling energy mechanism is one of the possible explanations (see “the photoionization/pooling model” part).¹⁹

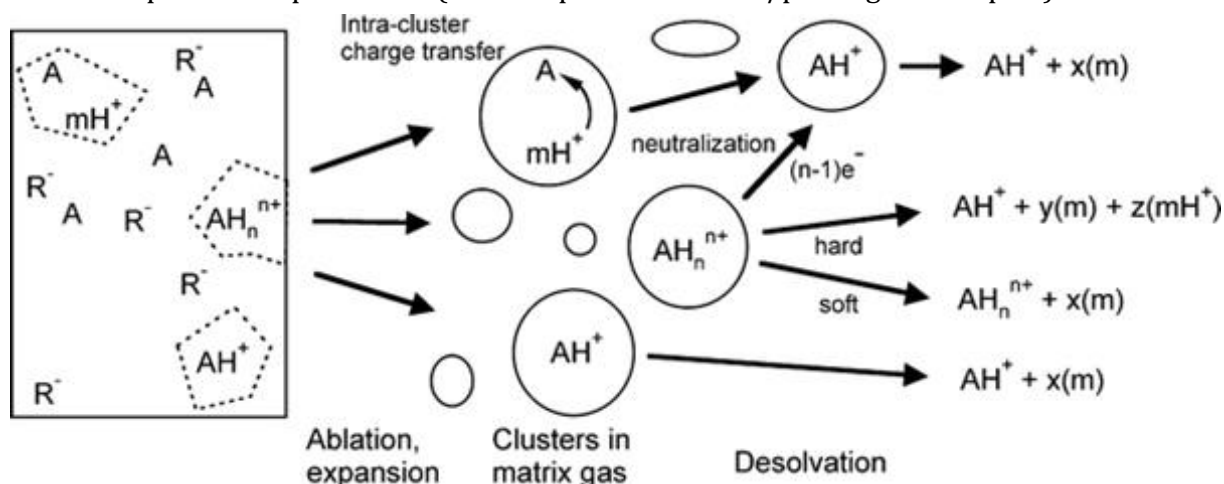


Figure III- 1. Schematic representation of the major processes proposed in cluster models of MALDI ionization. m = matrix, A = analyte, R⁻ = generic counter ion. Preformed ions, separated in the preparation solution, are contained in clusters ablated from the initial solid material. Some clusters contain a net excess of positive charge, others net negative (not shown). From ref.⁷

b) The photoionization/pooling model

This two-step model, also referred as the “Coupled Physical and Chemical Dynamics” (CPCD) model, has been extensively described by Knochenmuss as a quantitative model for MALDI predictions, based on the intra- and inter-molecular processes.^{14, 24-25}

It has been demonstrated that at least two sequential photons are required to allow photoionization of matrix molecules, under a laser operating at wavelength of 337 or 355 nm (i.e., 2-photon energies of 7.4 and 7.0 eV, respectively), commonly used in UV-MALDI. However, the typical MALDI laser photon fluxes are too low and do not allow for any significant resonant two-photon absorption to occur. As a consequence, a very efficient energy pooling between excited neighboring molecules in the crystals has been demonstrated.^{19, 26} This phenomenon involves the redistribution and concentration of electronic excitation energy of two nearby molecules, previously raised to the first excited

singlet state by photoexcitation (i.e. S_1 state, as shown Figure III- 2.c). However, as specified above, two photons from common N_2 or Nd:YAG lasers are not sufficient to generate ions from the pooling of two S_1 states; therefore the pooling of an S_1 with an S_n state is required (Figure III- 2.d).

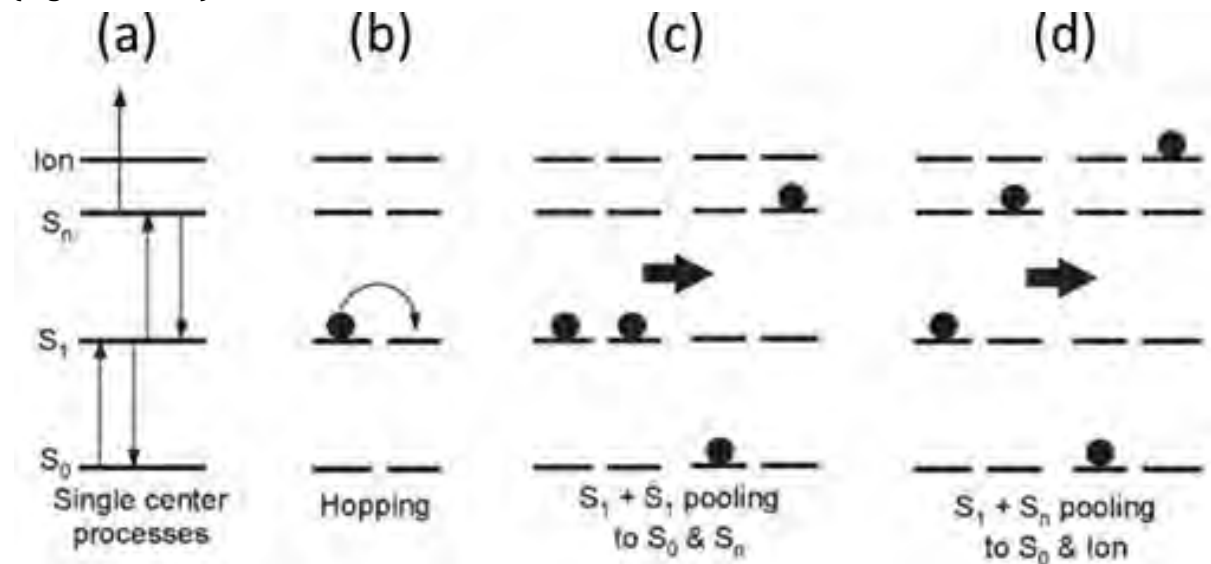


Figure III- 2. Schematic representation of the major processes proposed in photoexcitation/pooling model of MALDI ionization. (a) Unimolecular radiative processes. (b,c) Bimolecular processes showing: (b) exciton hopping; (c) pooling of two S_1 excitations, yielding an isoenergetic system consisting of a S_n + ground-state pair and (d) pooling of one S_1 and one S_n excitation, yielding a system consisting of an ion + ground-state pair. The excess energy above the ion state is converted to heat. From ref.^{7, 14, 24}

An example of the time-dependent evolution of a MALDI sample in the photoionization/pooling model is given Figure III- 3. Evidence for S_1 - S_n pooling was found in time-delayed 2-pulse MALDI experiments and a delayed maximum in matrix ion formation efficiency demonstrated that higher excited states are involved in ionization.²⁷ The consequence is that three types of matrix ions are generally visible in the mass spectra: radicals (m^+ , m^-), protonation/deprotonation products (mH^+ , $(m-H)^-$) and cation adducts (e.g. mNa^+).⁷

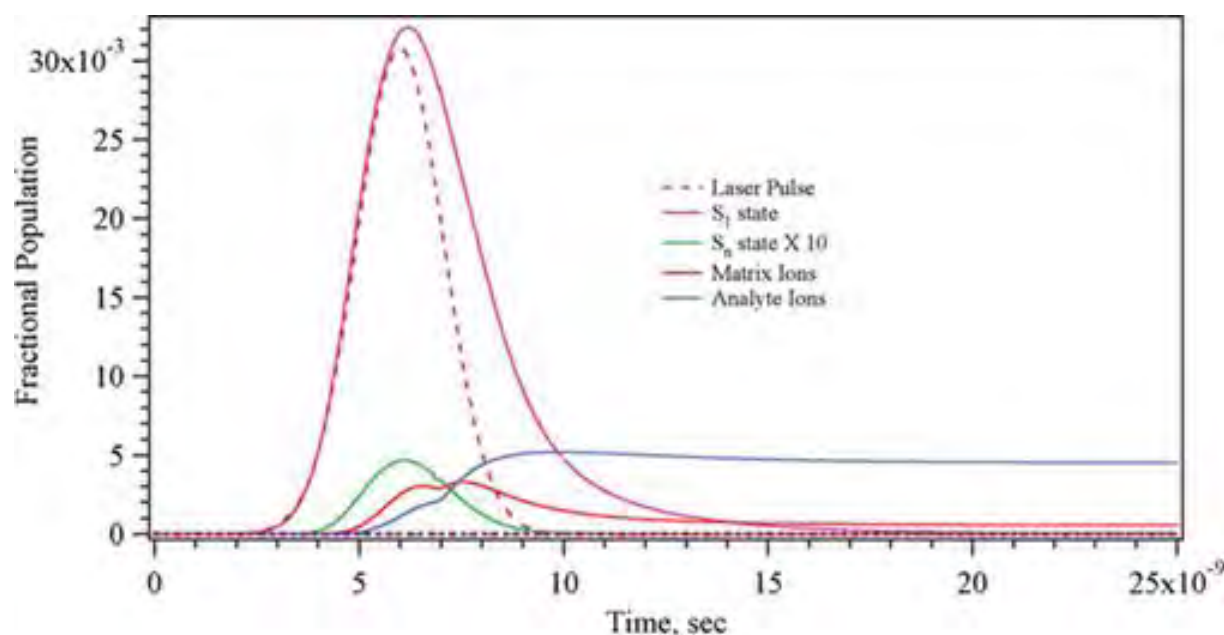


Figure III- 3. Example of the time-dependent evolution of a MALDI sample in the photoionization/pooling model. Pooling of abundant S_1 excitations leads to higher excited S_n matrix molecules. Pooling of S_1 and S_n leads to matrix ions (primary ionization). These react with analyte neutrals to yield analyte ions (secondary ionization), depleting the matrix ion signal. From ref.⁷

3.1.1.3. Ion formation in UV-MALDI (2): Secondary ionization mechanisms

Although the primary ionization events occurring are still discussed, the mechanisms for secondary ionization converge in favor of ion–molecule reaction thermodynamics and kinetics. Ion-ion and neutral(matrix)-ion chemical reactions occur and the charge in a cluster or particle may internally migrate via proton transfer, electron transfer or cationization to yield the most favorable ions.²⁸ Proton transfer neutralization to neutral species can also be responsible of the formation of the final gas-phase ions. Multiply charged analytes are usually difficult to create because of their internal electrostatic energy. This is more pronounced for low molecular weight compounds, but as larger compounds such as peptides or proteins have better ability to separate the charges, +2 or higher charge states can be formed.²⁸

In the case of singly charged clusters, neutralization leads to the formation of the singly charged analyte ions. Highly charged clusters experience do not survive in the plume due to the trapping of electrons (i.e. formed by matrix photoionization in the case of positive ions; by reaction with protonated matrix ions for negative ions).⁶ The most probable mechanism is electron capture dissociation. Hence, only a few portions of these ions are survivors and only singly charged are detected.⁶

It is noteworthy that matrix has to be considered as competitor of the analyte in the proton transfer process and thus the relative proton affinity of the matrix/analyte has to be considered. It has been recently demonstrated that matrix with lower proton affinity acts in favor of gas-phase protonation, as well as for peptides than for low molecular weight compounds.^{13, 21, 29-30}

3.1.1.4. Ion suppression effect

Ion suppression effect can be understood as a consequence of secondary reactions.^{7, 18, 31-33} The relative concentrations of analyte to matrix (matrix suppression effect, MSE) but also of analytes to each other (analyte suppression effect) have a significant effect on the resulting mass spectrum. The effect is understood as a competition for charges; if enough analyte is present, and its ions are thermodynamically favored, other ions will be depleted to insignificance.¹⁸ It was demonstrated that when analytes with molecular weights from 1'000 to 20'000 g/mol are mixed with matrix in relatively high molar ratio (10:1 to about 2000:1 matrix-to-analyte ratio), the appearance of positive matrix ions in the mass spectrum can be completely suppressed.³¹ This effect has also been shown for small molecules where MSE is used to produce high-quality MALDI mass spectra with strong analyte signals and weak or negligible matrix background peaks.³³⁻³⁴ The polarity of MSE is described as easily to predict, such as basic analytes strongly deplete protonated matrix so suppression occurs in positive mode.⁷ However, the MSE seems to be difficult to apply in a MS imaging workflow, where the matrix is preferably sprayed over the whole surface of the tissue (i.e. thus the exact amount of matrix deposited is difficult to determine) and the concentration of the analytes is unknown. The ion suppression effect is particularly challenging in MALDI-MS imaging approach. The analytes undergo ionization competition with other endogenous compounds (i.e. analyte suppression effect, ASE) present in the biological tissue, and that can exhibit higher ionization yield.³⁵ In other words, the response factor for each analyte is significantly dependent on the concentration of the others analytes, and is difficult to predict when dealing with complex biological tissues. Thus, the ion suppression is tissue specific, due to different chemical and biological composition, which reinforces the difficulty of performing quantification directly from tissue sections. This point will be discussed in the 5th Chapter of this manuscript.

3.1.2. MALDI on a triple quadrupole-linear ion trap (QqQLIT) and benefits for MS imaging of small molecules

3.1.2.1. Principle and features of the MALDI-QqQLIT platform

For performing (ultra)-fast MS (imaging) analysis of low molecular weight compounds, a platform combining a MALDI source, operated at intermediate pressure (i.e. 1 Torr), with a triple quadrupole-linear ion trap mass analyzer has been used (Figure III- 4).³⁶⁻³⁹ MALDI on a triple quadrupole decouples the desorption/ionization from the mass analysis,³⁷ as in delayed extraction (in axial TOF)⁴⁰⁻⁴¹ or using orthogonal MALDI-TOF.⁴² The elevated pressure in the source and in the pre-analyzer RF optics (Q_0 region), i.e. 1 Torr and 8 mTorr, respectively, allows collisional cooling of the ion beam, which reduces the ion fragmentation and allows to utilize high laser power. This is not possible with low-pressure TOF mass analyzers where the laser energy must be kept near the ionization threshold to avoid fragmentation.

In addition, this unique platform incorporates a high repetition rate laser (i.e. up to 1 kHz). This allows the production of a continuous beam of ions, compatible faster continuous scanning mode, such as the selected reaction monitoring available on a QqQ_{LIT} mass analyzer.^{1,43} Several studies report the beneficial effect on both the ion production and the rate of its formation, because high repetition rate lasers allow a large number of measurements to be gathered in a shorter amount of time, allowing rapid signal averaging and increasing sensitivity and reproducibility.³⁶⁻³⁸ In summary, high repetition rate lasers provide a much larger sample throughput compared to lower frequency lasers (i.e. 20-200 Hz) and increase the data quality.

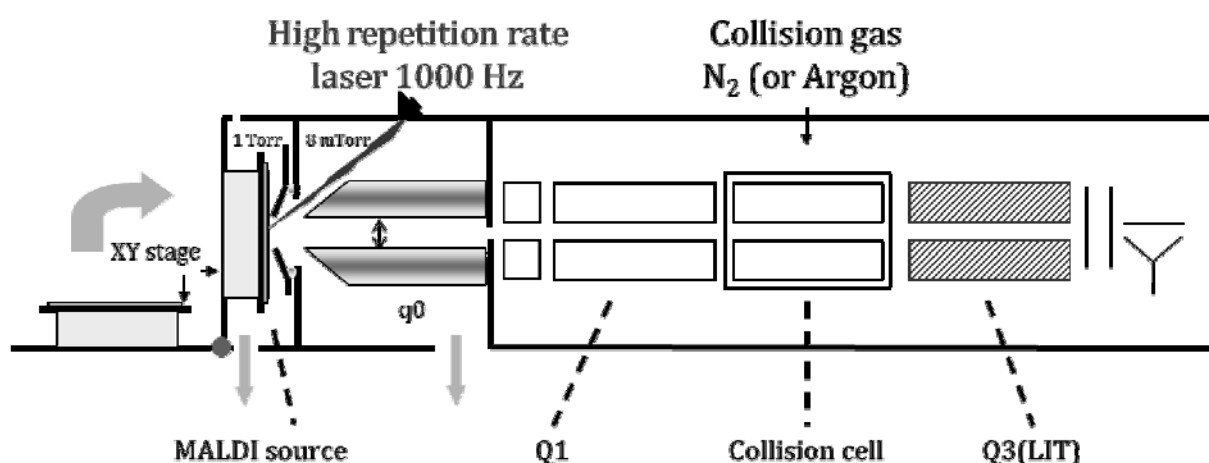


Figure III- 4. Schematic representation of the MALDI-QqQLIT platform used during this PhD work.

The main operating modes that have been used are the enhanced product ion (EPI) scan (Figure III- 5.a) and the selected reaction monitoring (SRM) modes (Figure III- 5.b). EPI offers the possibility of accumulating fragment ions in the third quadrupole operated in the linear ion trap mode. This allows for collecting structural information of the targeted analyte, in order to confirm the identity and thus the presence in the sample. The SRM mode, which provides a high level of selectivity and increased sensitivity by reducing chemical noise, consists in monitoring a specific reaction to a given analyte. Simultaneous SRM transitions can be monitored in a single experiment.

3.1.2.2. Benefits of the MALDI-QqQLIT platform for mass spectrometry imaging of small molecules

Tissues are complex systems, and the presence of numerous interfering peaks coming from endogenous metabolites, together in combination with abundant interfering matrix-derived ions in the low m/z region may hamper the identification of low molecular weight compounds (e.g. pharmaceuticals, drugs of abuse and their metabolites).^{4, 14, 44} To deal with that issue and to provide sufficient analytical selectivity and sensitivity, various strategies have been developed. In particular, quadrupole and ion trap mass analyzers are used for targeted analysis of small molecules. Although they do not provide enough mass resolution to resolve analyte peaks from endogenous species peaks, they offer tandem MS capabilities

to allow for generating structural information for compound identification or confirmation as specified in the previous paragraph. The SRM mode offers the highest level of sensitivity, where a specific precursor ion to one (or several) product ion transition(s) is monitored.^{1-2, 43, 45}

Imaging experiments can benefit from the fast duty cycle of the SRM approach (i.e. down to 10 ms per transition, including 5 ms-pause between transitions) and together with optimized instrumental parameters (i.e. high sample stage velocity in rastering mode, high laser frequency), it can generate in a relatively rapid fashion whole body section imaging for instance.¹ Playing with all those parameters can also influence the resolution of the generated image, as described more in details in Chapter 5, Part 2.

The MALDI-SRM/MS approach has been proven quantitative in several studies^{39, 46-50} and therefore is expected to be a suitable platform for the targeted imaging and quantification of low molecular weight compounds (i.e. pharmaceuticals, drugs of abuses, metabolites) directly from tissue sections.

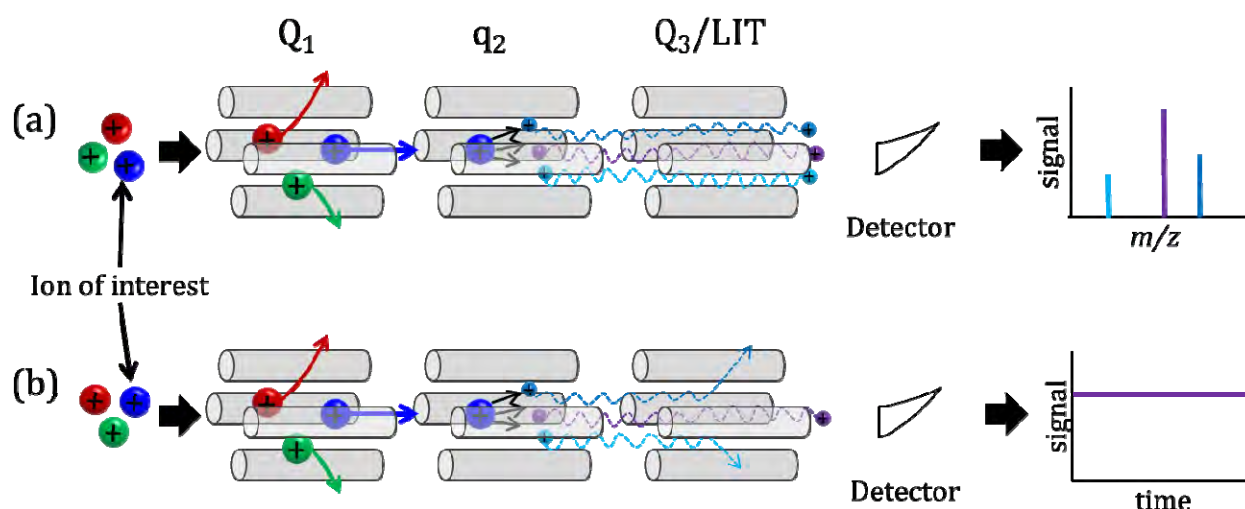


Figure III- 5. Principles of the (a) enhanced product ion (EPI) scan and (b) selected reaction monitoring (SRM) mode operated on the QqQ_{LIT} mass analyzer. EPI allows for collecting fragment ions (in LIT mode) from previously isolated precursor ion (in the first quadrupole Q₁), providing structural information of the isolated compound. SRM allows for monitoring a specific precursor ion (fixed m/z in Q₁) → fragment ion (fixed m/z in Q₃) transition for each analyte.

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 2 – Alternative CHCA-based matrices for the analysis of low molecular weight compounds by UV-MALDI-tandem mass spectrometry

Adapted from:

Porta T., Grivet C., Knochenmuss R., Varesio E. and Hopfgartner G., Alternative CHCA-based matrices for the analysis of low molecular weight compounds by UV-MALDI-tandem mass spectrometry. *J. Mass Spectrom.*, **2011**, 46:144-152

Abstract

Analysis of low molecular weight compounds (LMWC) in complex matrices by vacuum matrix-assisted laser desorption/ionization (MALDI) often suffers from matrix interferences, which can severely degrade limits of quantification. It is therefore useful to have available a range of suitable matrices, which exhibit complementary regions of interference. Two newly synthesized α -cyanocinnamic acid derivatives are reported here; (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid (NpCCA) and (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid (AnCCA). Along with the commonly used α -cyano-4-hydroxycinnamic acid (CHCA), and the recently developed 4-chloro- α -cyanocinnamic acid (Cl-CCA) matrices, these constitute a chemically similar series of matrices covering a range of molecular weights, and with correspondingly differing ranges of spectral interference. Their performance was compared by measuring the signal-to-noise ratios (S/N) of 47 analytes, mostly pharmaceuticals, with the different matrices using the selected reaction monitoring (SRM) mode on a triple quadrupole instrument equipped with a vacuum MALDI source. AnCCA, NpCCA and Cl-CCA were found to offer better signal-to-noise ratios in SRM mode than CHCA, but Cl-CCA, yielded the best results for 60% of the compounds tested. To better understand the relative performance of this matrix series, the proton affinities (PA) were measured using the kinetic method. Their relative values were: AnCCA > CHCA > NpCCA > Cl-CCA. This ordering is consistent with the performance data. The synthesis of the new matrices is straightforward and they provide i) tunability of matrix background interfering ions, ii) and enhanced analyte response for certain classes of compounds.

Key words

CHCA-based matrices, low molecular weight compounds, MALDI-SRM/MS, experimental proton affinities determination, mass spectrometry

3.2.1. Introduction

Since its introduction in the late 1980s,^{3-4, 51} matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been widely used for polymer, protein and peptide analysis, mostly in combination with time-of-flight mass analyzers (TOF, Q-TOF, TOF/TOF).⁵² MALDI has recently been extended to the qualitative or quantitative analysis of low molecular weight compounds (LMWC),^{1, 33, 50, 53-54} but the presence of abundant interfering matrix-derived ions in the low m/z region remains a significant problem.^{4, 14, 44} Various strategies to deal with this have been developed, including use of the matrix suppression effect,³³ high molecular weight matrices,⁵⁵⁻⁵⁶ ionic (liquid) matrices,⁵⁷⁻⁵⁹ proton sponges,⁶⁰⁻⁶¹ matrix-free desorption from solid substrates,⁶²⁻⁶³ and high resolution MS.⁵³

With quadrupole instruments, the matrix interference issue is especially serious because these mass analyzers operate at low, often unit, mass resolution. A typical example is the analysis of talinolol in microsomes, where the signal of several hydroxylated metabolites ($[M+H]^+$ m/z 380) of talinolol ($[M+H]^+$ m/z 364) overlap with the ^{13}C isotopes of the protonated CHCA dimer ($[2M+H]^+$, m/z 379), making this matrix unsuitable for the analysis of talinolol metabolites. Other matrices, such as 2,5-dihydroxybenzoic acid or sinapinic acid matrices could be used, but resulted in a loss of signal sensitivity in this case.⁶⁴

Although no one matrix is optimal for all analytes, α -cyano-4-hydroxycinnamic acid (CHCA) has been successfully used for quantitative MALDI analysis of low molecular weight compounds by selected reaction monitoring (SRM) mode.^{1, 39, 49, 65} CHCA is a good initial matrix to try, if an interference-free transition is available.^{36, 38-39} In SRM mode and with singly charged ions it is quite rare that an isobaric interference produce the same fragment. However, with MALDI the interferences do not come from the sample but from the matrix which is present at high level which mainly affect the limit of detection of many transitions. If no such transition is possible, the matrix must be changed, but it would be desirable to retain as many of the favorable properties of CHCA as possible. Here we propose and test new matrices which differ from CHCA only in the size of the aromatic ring system. This shifts interferences to higher m/z ranges, but retains much of the chemical functionality of CHCA.

The new matrices are tested in SRM mode on a triple quadrupole linear ion trap (QqQ_{LIT}). The response (signal-to-noise ratio) of 47 different analytes was compared to CHCA, and to 4-chloro- α -cyanocinnamic acid (Cl-CCA). The latter was recently introduced by Jaskolla *et al.*, and has mostly been used for medium or high molecular weight compounds. It was particularly successful for peptide analysis.^{13, 29, 66} Together, these four compounds constitute a series of chemically similar matrices which cover a range of molecular weights, and have different interference patterns.

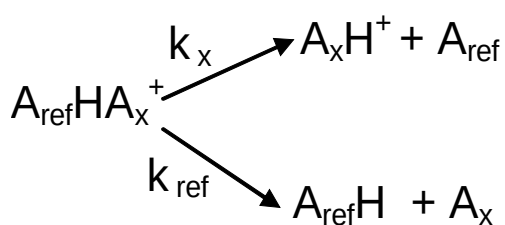
In addition to empirical investigations of matrix performance, fundamental MALDI-relevant properties of each were measured. The solid-phase absorption spectrum determines how efficiently energy is deposited by the laser, and whether a matrix is suitable for a particular excitation wavelength. When analyte ions are formed by secondary proton transfer reactions from primary matrix ions^{4, 7} the gas-phase proton affinities play a key role in

determining the rate and extent of reaction, and hence signal. The proton affinities of the new matrices were determined using the Cooks' kinetic method.⁶⁷

3.2.2. Theory: the kinetic method for the determination of the proton affinity of MALDI matrices

The kinetic method is used for measurement of the proton affinity (PA) values of common and synthesized MALDI matrices.⁶⁸⁻⁷¹ The measurement is carried out under electrospray ionization where proton-bound dimers are generated between a first compound, which is a reference compound A_{ref} , and a second compound of interest A_x of which PA has to be determined.

The difference between the gas-phases basicities of two neutral molecules (i.e. the difference between the free energies of protonation) can be deduced from the MS/MS spectrum of the proton-bound dimer incorporating the two molecules. Their dissociation can be studied upon collision induced dissociation (CID) performed on a triple quadrupole mass analyzer. The method consists in the isolation of the precursor ion (i.e. proton-bound dimers where two molecules are loosely bound to a central proton) in the first quadrupole and then, in the induction of its dissociation by collision with a neutral gas molecule (e.g. nitrogen, helium) in the collision cell. The collisional activation give two fragments ions, namely the protonated forms of each of the molecules which comprise the dimer ion, and the only two processes observed can be represented as shown Scheme III- 1 (adapted from McLuckey *et al.*⁶⁹).



Scheme III- 1. CID dissociation of a proton-bound dimer $[A_{ref}HA_x]^+$, formed by: A_{ref} : reference compound with known proton affinity; A_x : here, the MALDI matrix with unknown PA. k_{ref} and k_x : rates of competitive dissociations. I_{ref} and I_x : intensities of the signals given by $A_{ref}H^+$ and A_xH^+ , respectively, in the CID spectrum of $A_{ref}HA_x^+$.

k_{ref} and k_x are the rates of competitive dissociations that determine the intensity I_{ref} and I_x of the signals given by $A_{ref}H^+$ and A_xH^+ respectively in the fragmentation spectrum of $A_{ref}HA_x^+$. When reverse activation energies and also the frequency factor (which determine k_{ref} and k_x) cancel, it becomes possible to find the difference in PA of A_{ref} and A_x which is given by the simple Equation 1:

$$\Delta PA = R \cdot T_{eff} \cdot \ln \left(\frac{I_{ref}}{I_x} \right) \quad \text{Equation (1)}$$

with: R : the gas constant; and T_{eff} : the effective temperature of the activated dimer ion. $R \cdot T_{eff}$ reflects the internal energy of the dimer ion. Under these conditions, the compound with the greater PA gives the largest peak in the MS/MS spectrum of the proton-bound dimer.⁶⁹ Equation can also be express as follow:

$$\ln \left(\frac{I_{ref}}{I_x} \right) = [PA(A_{ref}) - PA(A_x)] \times R \cdot T_{eff} \quad \text{Equation (2)}$$

Based on previously established work, a linear kinetic plot can be built (i.e. the so-called “Cooks plot”), traducing the direct relation between $\ln\left(\frac{I_{ref}}{I_x}\right)$ in function of the proton affinity of reference compounds. As a result, the interception with the x-axis gives directly the proton affinity value of the compound of interest.⁶⁹

3.2.3. Experimental

3.2.3.1. Chemicals

Unless otherwise stated, all compounds were commercially available and used without any further purification. Methanol (MeOH, HPLC grade), acetonitrile (ACN), adenosine triphosphate (ATP), hydrocortisone, L-cysteine (Cys), creatine, 2-naphthaldehyde, L-glutathione (Gly), guanosine, thymine, adenine, deoxyuridine, cytidine, L-tryptophan (Trp), L-tyrosine (Tyr), uracil, fipexide hydrochloride, testosterone, reserpine, estrone, dimethylsulfoxide-d6 (DMSO-d6) were purchased from Sigma-Aldrich (Buchs, Switzerland). α -lactose was purchased from Supelco (Buchs, Switzerland). 2-arachidonyl-glycerol (2-AG) was purchased from Cayman Chemicals (Michigan, USA). Creatinine was purchased from Alfa Aesar (Karlsruhe, Germany). 17-hydroxyprogesterone (17-OH-progesterone) was purchased from Riedel-de-Haen (Buchs, Switzerland). L-phenylalanine (Phe), L-proline (Pro), aldosterone, α -cyano-4-hydroxycinnamic acid (CHCA), caffeic acid, sinapinic acid, anthracene-9-carbaldehyde, *tert*-butyl 2-cyanoacetate, triethylamine (TEA) and acetamide were purchased from Fluka (Buchs, Switzerland). Toluene, 3-aminobenzonitrile, 3-bromoaniline and 3-fluoroaniline were provided by Acros Organics (New Jersey, USA). 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (GPCho) was supplied by Avanti Polar Lipids (Instrumenchemie BV, Delfzyl, Netherlands). Amprenavir (APV), atazanavir (ATV), chloroprothixene, clonazepam, everolimus, fluoxetine, fluphenazine, indinavir (IDV), levodopa, lopinavir (LPV), methaqualone, nelfinavir (NFV), nordiazepam, norfluoxetine, nortriptyline, risperidone and its oxidative metabolite hydroxyl-risperidone (risperidone-OH), ritonavir (RTV), saquinavir (SQV) and venlafaxine were a gift from the Geneva University Hospital (Dr. Fathi, Geneva, Switzerland). Talinolol and propranolol hydrochloride were obtained from the Clinical Toxicology and Pharmacology Department of Basel Hospital (Basel, Switzerland). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

3.2.3.2. Synthesis of alternative solid CHCA based matrices

4-chloro- α -cyanocinnamic acid (Cl-CCA), (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid (NpCCA) and (2E)-3-(anthracen-9-yl)-2-cyanoprop-2enoic acid (AnCCA) were synthesized in two steps. The first step was a proline catalyzed Knoevenagel reaction using *ter*-butyl 2-cyanoacetate and substituted aldehydes (2-naphthaldehyde, anthracene-9-carbaldehyde, 4-chloro benzaldehyde). The resulting solids consisted of substituted *tert*-butyl 2-cyanoacrylate intermediates. The second step was a hydrolysis with TFA. (E)-*ter*-butyl 2-

cyano-3-(naphthalen-2-yl)acrylate and (E)-tert-butyl-3-(anthracen-9-yl)-2-cyanoacrylate were respectively added to TFA in a round-bottomed flask to give a yellow solution. (E)-tert-butyl 3-(4-chlorophenyl)-2-cyanoacrylate and TFA gave a colorless solution. The reactions were stirred with a magnetic stir bar for 45 min, and precipitated. TFA was evaporated twice with toluene. The residues were triturated with water, filtered through a Buchner funnel, washed with water, and collected. The resulting solids (NpCCA, AnCCA and Cl-CCA) were then recrystallized from MeOH and dried in oven at 40°C under vacuum. Yields were 67.2 %, 64.0 % and 77 % for NpCCA, AnCCA and Cl-CCA respectively. The accurate mass determination by ESI-TOF MS and the structural NMR confirmation for the synthesis of MALDI matrices are detailed Table III- 1.

Table III- 1. Accurate mass determination by ESI(+)-TOF MS (QSTAR XL, AB Sciex) and structural NMR confirmation for the synthesis of MALDI matrices

	Accurate mass determination			¹ H-NMR (300 MHz, DMSO-d ₆)
	observed m/z	expected m/z	accuracy	observed δ (ppm)
NpCCA [M+H] ⁺	224.0715	224.0706	4.0 ppm	14.05 (s, 1H), 8.57 (s, 1H), 8.49 (s, 1H), 8.22 (dd, ³ J = 8.7 Hz, ⁴ J = 1.6 Hz, 1H) 8.09 (d, J = 8.7 Hz, 1H), 8.02 (m, 2H), 7.8 (m, 2H)
AnCCA [M+H] ⁺	274.0867	274.0862	1.8 ppm	14.29 (s, 1H), 9.27 (s, 1H), 9.03 (s, 1H), 8.22 (m, 2H), 8.02 (m, 2H), 7.68 (m, 4H)
Cl-CCA [M+Na] ⁺	229.9983	229.9979	1.7 ppm	14.05 (s, 1H), 8.35 (s, 1H), 8.06 (d, 2H, J = 8.6 Hz), 7.68 (d, 2H, J = 8.6 Hz)

3.2.3.3. Analytes tested

A set of 47 compounds with molecular weights from 110 to 960 g/mol were tested. The pKa for acids ranged from 0.8 to 18.11 and the pKa of protonated bases from -4.19 to 13.51. The hydrophobicity, given by the calculated log D (clog D), ranged from -8.45 to 12.53 at pH = 3 and from -4.64 to 12.54 at pH = 8. It includes analytes such as steroids, phospholipids, sterols, amino acids, nucleotides, nucleosides, nitrogen bases and other drug compounds. Table S1 in supporting material shows the solution-phase physicochemical properties of the analytes calculated using SciFinder Scholar and the ACD/Labs software suite (version 8.14, Toronto, Canada).

3.2.3.4. MALDI sample preparation

Saturated matrix solutions were prepared by dissolving 30 mg of CHCA, 8 mg of NpCCA or 8 mg of AnCCA in 1 mL of methanol:water (4:1, v:v) with 0.1 % formic acid (FA). More than 50% water resulted in the precipitation of the NpCCA and AnCCA matrices. Due to the high solubility of Cl-CCA in this solvent mixture (more than 100 mg/mL), the proportion of organic solvent was reduced to 60% in order to decrease the saturation limit. In this solvent, a 30 mg/mL solution of Cl-CCA was saturated. The solutions were sonicated for 10 minutes, centrifuged for 5 minutes and the supernatant was used for analysis.

Stock solutions of all analytes were prepared in appropriate solvents at a concentration of 1 mg/mL and stored at 4°C. All amino acids, nucleosides, nucleotides, purines, pyrimidines were dissolved in water. Steroids were dissolved in methanol, fatty acids in ethanol, and phospholipids in chloroform. Other pharmaceuticals were dissolved in methanol. These solutions were then diluted 200-fold to a final concentration of 5 ng/ μ L in methanol:water (1:1, v:v). Sample solutions were then mixed 1:1 with saturated matrix and 1 μ L of this mixture was spotted manually onto a stainless steel 384-well MALDI target (Opti-TOF™ 384 Well Insert (123 x 81 mm), AB/MDS Sciex, Concord, ON). Blank samples were obtained by mixing the saturated matrix solutions with the same volume of methanol:water (1:1, v:v).

3.2.3.5. UV absorption measurements

Solid-phase UV-Vis absorption spectra were acquired on a Lambda 900 UV-Vis-NIR spectrometer (Perkin Elmer) operating in reflectance mode. Spectra were acquired from 200 to 600 nm.

Solution-phase UV spectra (Figure III- 6) were acquired on a Lambda 20 UV-Vis spectrophotometer (Perkin Elmer, Massachusetts, USA / Cells made of quartz glass, SUPRASIL®, type 100-QS, path length = 10 mm). The laser wavelengths typically used in MALDI, 337 nm and 355 nm, are marked with vertical dotted lines. The molar extinction coefficients (ϵ) were calculated ($n = 6$) at 337 nm and 355 nm according to the Lambert-Beer law from matrix solutions at concentrations of 50 μ M (dissolved in 1.3 % MeOH) and 30 μ M (in 0.8 % MeOH) and are noticed in the table below.

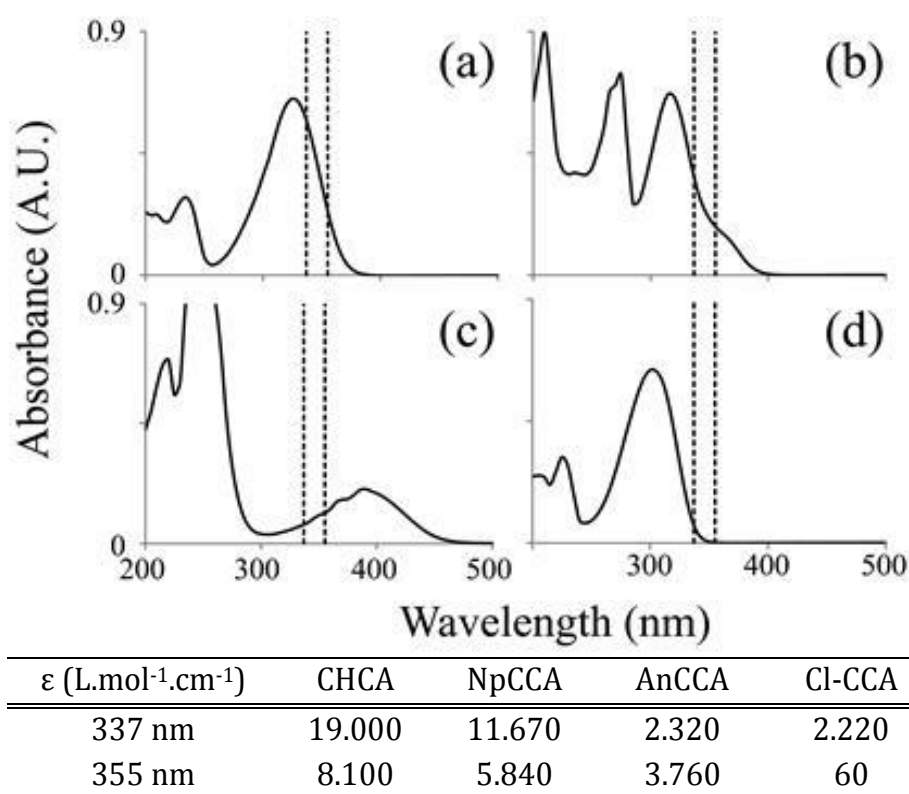


Figure III- 6. Solution-phase UV spectra of the matrices: (a) CHCA; (b) NpCCA; (c) AnCCA and (d) Cl-CCA and molar extinction coefficients (ϵ) were calculated ($n = 6$) at 337 nm and 355 nm.

3.2.3.6. MALDI- QqQ_{LIT} mass spectrometry

MALDI mass spectra were acquired in positive polarity on a prototype triple quadrupole linear ion trap 4000 QTRAP mass spectrometer (AB/MDS Sciex, Concord, ON). A frequency-tripled Nd:YAG laser was used (355 nm, 0.5 ns pulse duration, 1000 Hz repetition rate). The laser spot on the sample has an elliptical shape of approximately 200 μm by 100 μm (corresponding to an area of $6.28 \times 10^{-4} \text{ cm}^2$). Prior the analysis, the laser energy was calibrated using a power/energy meter (EPM1000, Coherent, Portland, OR) equipped with a pyroelectric energy sensor (J25LP-3A, Coherent, Portland, OR). Subsequently, the laser energy was set using the M3Q Server software. The MALDI source was pressurized with nitrogen gas and the final pressures in the target plate and Q_0 regions were 1 Torr and 8 mTorr respectively. Further parameters were set as following: entrance potential (EP) 10 V, collision cell exit potential (CXP) 20 V, ion energies for Q_1 and Q_3 were 1 and 0.8 V respectively. Unit resolution was used in both quadrupoles. The Analyst 1.5 software (AB/MDS Sciex) was used for the control of the mass spectrometer and for data acquisition. The MALDI source and its laser were controlled using a prototype software package (M3Q Server, ver. 1.0b16 AB/MDS Sciex). The laser energy was optimized for each matrix and each analyte (between 0.5 to 6.5 $\mu\text{J}/\text{pulse}$). The average of the optimum laser energy obtained for each analyte was calculated for each matrix. For the Q_1 full scan mode (m/z 100-1000 at 500 u/s), data were collected using the discrete mode and resulted in the sum of 15 spectra. For the selected reaction monitoring mode (dwell time = 50 ms), data were collected using the line scan mode at a fixed laser frequency (*i.e.* 1000 Hz) and a constant moving speed of the target plate of 0.5 mm/s. Nitrogen was used as collision gas (pressure in $q_2 = 2.8 \times 10^{-5}$ Torr). The monitored transitions were optimized for each analyte and are listed in Table III- 2.

Table III- 2. SRM transitions monitored in positive ionization mode by flow injection analysis. The collision energy (eV) is indicated in parenthesis. *: Sodium adduct [M+Na]⁺ was used as precursor ion to determine the most intense SRM transition corresponding to the analyte.

Analyte	SRM transition	Analyte	SRM transition
17-OH-Progest.	331.2→97.0 (35)	L-Glutathione	308.1→179.1 (20)
2- Arachidonyl-Glycerol	379.4→283.3 (25)	L-Phenylalanine	166.0→120.1 (20)
α-Lactose*	365.0→347.0 (29)	L-Proline	116.1→70.0 (25)
Adenine	136.0→119.0 (30)	L-Tryptophan	205.0→188.1 (15)
Aldosterone	361.3→343.2 (29)	L-Tyrosine	182.1→165.3 (15)
Amprenavir	506.3→418.1 (20)	Levodopa	198.1→181.1 (15)
Atazanavir	705.8→168.2 (60)	Lopinavir	629.8→155.5 (60)
ATP	508.1→136.2 (40)	Methaqualone	251.2→132.3 (45)
Chlorprothixene	316.0→270.8 (30)	Nelfinavir	568.3→135.5 (60)
Clonazepam	316.2→270.0 (35)	Nordiazepam	271.0→140.0 (45)
Creatine	132.1→90.3 (20)	Norfluoxetine	296.1→132.1 (15)
Creatinine	114.1→86.1 (20)	Nortriptyline	264.0→91.3 (30)
Cytidine	244.1→112.1 (20)	Propranolol	260.0→116.3 (30)
Deoxyuridine	229.0→113.0 (15)	Reserpine	609.7→195.2 (60)
Estrone	271.2→253.3 (30)	Risperidone	411.4→191.3 (35)
Everolimus*	980.6→980.6 (25)	Risperidone-OH	427.2→207.2 (35)
Fipexide	389.1→135.0 (25)	Ritonavir	724.6→296.5 (25)
Fluoxetine	310.2→148.0 (15)	Saquinavir	671.5→570.4 (20)
Fluphenazine	438.3→143.0 (45)	Talinolol	364.2→308.2 (15)
GPCho	762.7→184.0 (48)	Testosterone	289.1→97.1 (30)
Guanosine	284.1→152.1 (30)	Thymine	127.0→109.9 (30)
Hydrocortisone	363.1→251.0 (26)	Uracil	113.0→96.0 (30)
Indinavir	614.6→421.0 (45)	Venlafaxine	278.0→260.3 (20)
L-Cysteine	122.1→76.1 (19)		

3.2.3.7. Determination of the proton affinities of MALDI matrices by ESI-MS/MS

a) Sample preparation

For the determination of the PA of CHCA, NpCCA and AnCCA, solutions containing MALDI matrices and reference compounds were prepared at a concentration of 1 mM in MeOH/H₂O/HCOOH (75:25:0.1). For the determination of the PA of the chlorinated matrix, sinapinic acid and caffeic acid, solutions were prepared in ACN/H₂O/HCOOH (50:50:1). The formation of the dimers containing Cl-CCA and CHCA was optimum at a concentration of Cl-CCA 10 fold the concentration of CHCA. The proton affinities of MALDI matrices were determined with the following references: CHCA: 3, 5-7; SA: 7-10; Caffeic acid: 5, 6, 9, 10; NpCCA: 3-7; AnCCA: 3-7; Cl-CCA: 1-4 (see Table III- 3). The molecular structures of the references are presented Scheme III- 2.

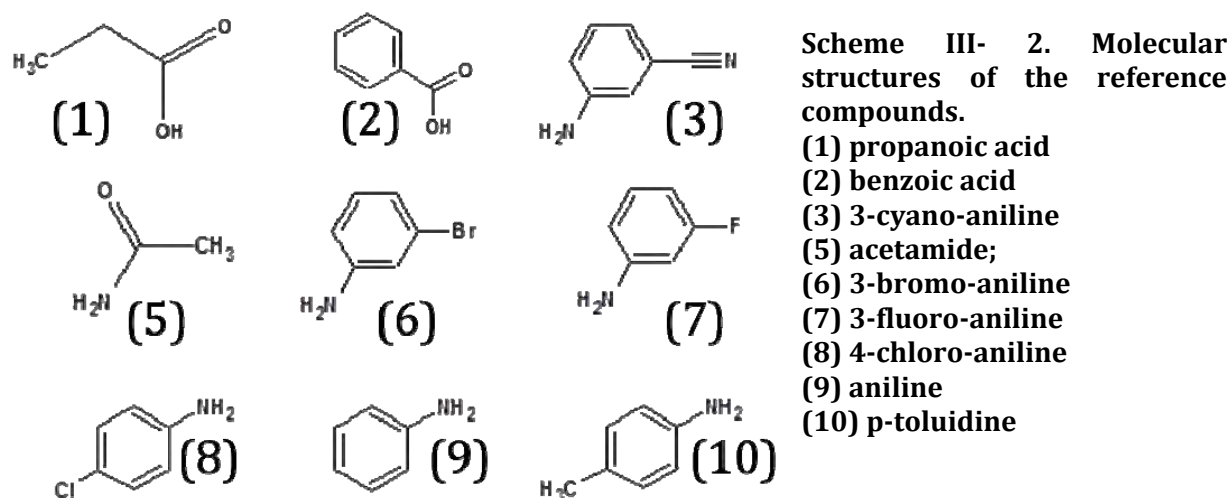


Table III- 3. Proton affinities of reference compounds found in the literature. * From <http://webbook.nist.gov>

Number	Reference	PA (kJ/mol)
1	Propanoic acid	797.2*
2	Benzoic acid	821.1*
3	3-Cyano-Aniline	839.3, 842.8 ⁷²
4	CHCA	841.5, ⁷¹⁻⁷² 841.0, ⁷¹ 766.2 ⁷³
5	Acetamide	864.2 ⁷²
6	3-Bromo-Aniline	870.3
7	3-Fluoro-Aniline	870.7, 872.1 ⁷²
8	4-Chloro-Aniline	876.5
9	Aniline	884.5
10	p-toluidine	896.2 ⁷¹

b) Instrumentation of infusion-ESI-SRM/MS

MS spectra acquisition was performed in positive polarity on a triple quadrupole linear ion trap 4'000 QTRAP mass spectrometer (AB/MDS Sciex, Concord, ON) fitted with electrospray ionization (ESI) source. Samples were infused at a flow rate of 15 $\mu\text{L}/\text{min}$. 3-cyanoaniline (PA = 839.3 kJ/mol⁷¹), acetamide (PA = 864.2 kJ/mol⁷²), 3-bromoaniline (PA = 870.3 kJ/mol⁷¹) and 3-fluoroaniline (PA = 870.7 kJ/mol⁷¹) were chosen as reference compounds for the PA determination of CHCA, NpCCA and AnCCA. Propanoic acid (PA = 797.2 kJ/mol), benzoic acid (PA = 821.1 kJ/mol), 3-cyanoaniline and CHCA were the reference compounds for the Cl-CCA measurements. The monitored transitions were $A_{\text{ref}}\text{HA}_x^+ \rightarrow A_{\text{ref}}\text{H}^+$ for the reference compound as product ion in the MS/MS spectrum and $A_{\text{ref}}\text{HA}_x^+ \rightarrow A_x\text{H}^+$ for the matrix with unknown proton affinity. Declustering potential was set on 10 V to prevent in-source dissociation of the proton-bound dimer. Collision energy varied from 5 to and 10 eV (step of 0.1 V) for each transition and collision induced dissociation (CID) was performed using nitrogen as collision gas (vacuum gauge in q2 was 3.6e-5 Torr with a CAD=4). The

spray voltage was of 4.8 kV; the pressure of the curtain gas was of 15 psi. The analyzer resolution was unit for both Q_1 and Q_3 .

3.2.3.8. Data processing

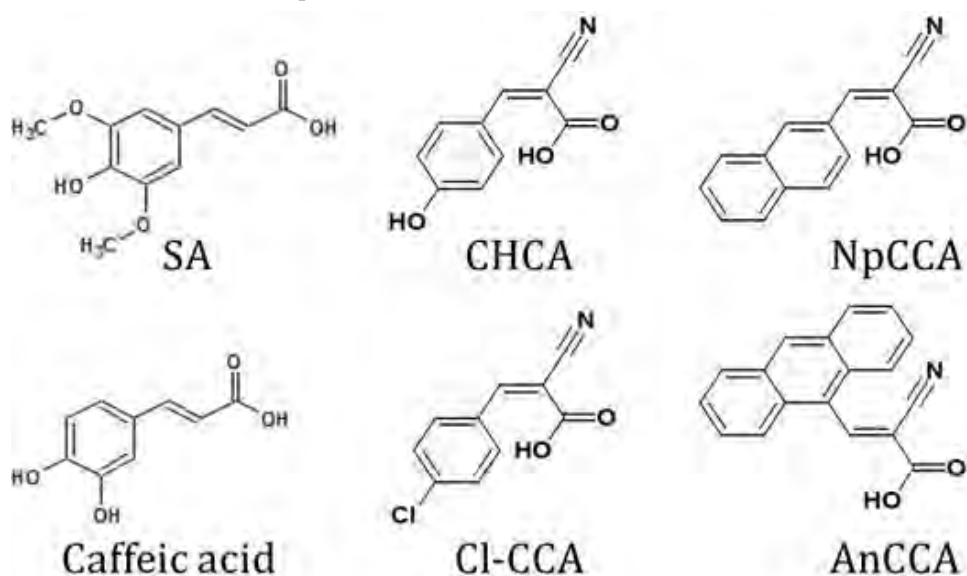
Data were processed with the MaldiQuan software (v. 1.4.1, AB/MDS Sciex). The spot-to-spot reproducibility of the signal was calculated using the area under the relevant SRM trace. The signal-to-noise ratio was determined by dividing the maximum SRM signal height measured in a spot containing the analyte by the maximum SRM signal height from an adjacent spot containing only the matrix. Because MALDI samples normally exhibit considerable intrinsic variability, only S/N values differing by more than 30% were considered to be significantly different.

3.2.4. Results and Discussion

3.2.4.1. Characterization of the investigated matrices

a) Physicochemical properties of the synthesized matrices

The hydrophilic or hydrophobic nature of a MALDI matrix, as reflected in its solution-phase log D value, has been suggested by some authors to be useful for predicting incorporation of analytes in the matrix crystals.^{39, 47, 49} CHCA has the lowest calculated log D value of these matrices considered here, $\log D_{\text{pH}=3} = 1.53$. The others are Cl-CCA (2.59), NpCCA (3.29), and AnCCA (4.53). This suggests that NpCCA and AnCCA might be somewhat better suited for analysis of lipophilic compounds than CHCA. We will return to this point below. The physical properties of the matrices are presented Table III- 4 and their structures Scheme III- 3.



Scheme III- 3. Molecular structures of MALDI matrices. SA: sinapinic acid; CHCA: α -cyano-4-hydroxycinnamic acid; Cl-CCA: 4-chloro- α -cyanocinnamic acid; NpCCA: (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid and AnCCA: (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid.

Table III- 4. Calculated physicochemical properties of the different matrices. These properties are calculated using the ACD/Labs software suite (version 11.01, Toronto, Canada).

Matrix	CHCA	Sinapinic acid	Caffeic acid	NpCCA	AnCCA	Cl-CCA
Molecular formula	$C_{10}H_7NO_3$	$C_{11}H_{12}O_5$	$C_9H_8O_4$	$C_{14}H_9ON_2$	$C_{18}H_{11}ON_2$	$C_{10}H_6ClNO_2$
MW (g/mol)	189.18	224.21	180.16	223.23	273.29	207.61
Log P	1.53	1.286	1.424	3.29	4.53	2.59
Log D (pH3)	-0.3	1.24	1.39	1.25	2.48	0.41
Log D (pH8)	-2.23	-2.28	-2.12	-0.46	0.78	-1.16
pKa	9.58 / 1.17	3.98	4.04	0.95	0.95	0.81

b) Matrix crystallization behavior

Uniform crystallization behavior is important for avoidance of so-called “hot spots”. These can hamper the quality of quantitative analysis by greatly increasing in-sample signal variability.^{58, 74} Thus, the dried droplet method was used for the sample preparation, since it has been successfully applied for automation in high-throughput quantitative analysis.^{58, 74} As a result, CHCA, NpCCA, and AnCCA gave mostly small crystals, uniformly distributed and leading to acceptable spots, whereas Cl-CCA was more heterogenic than the other matrices, as illustrated in Figure III- 7.

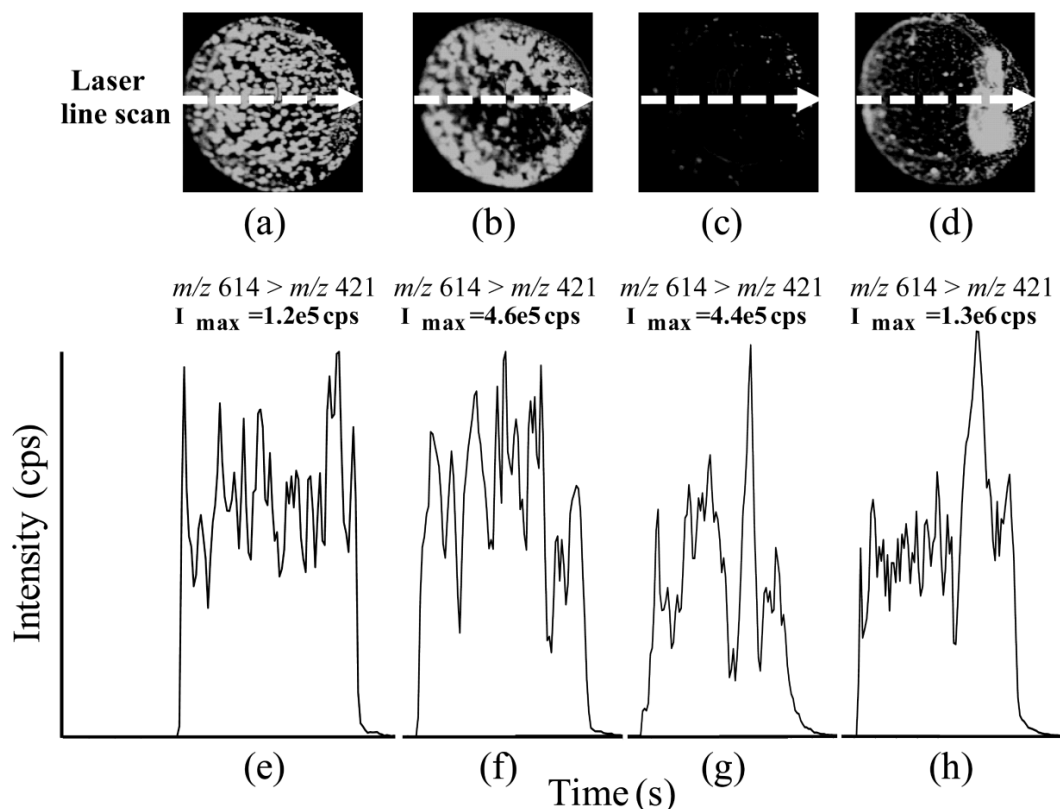


Figure III- 7. 1 μ L-dried drop MALDI samples (a-d) containing indinavir (5 ng/ μ L) mixed 1:1 with each saturated matrix solutions, and the corresponding SRM traces (e-h); (a,e) CHCA (2.0 μ J/pulse); (b,f) NpCCA (2.1 μ J/pulse); (c,g) AnCCA (3.6 μ J/pulse) and (d,h) Cl-CCA (3.0 μ J/pulse). The diameter of all spots was approximately 2 mm. Each acquisition was performed in line scan mode, as indicated by the dashed lines drawn across the spots.

Figure III- 7 also shows line scans of the SRM signal of the anti-retroviral indinavir (IDV), across the spots. The signal largely reflects the optical density of the sample. As a result, the AnCCA and Cl-CCA spots displayed the most pronounced "hot spot" effects. Spot-to-spot reproducibility based on absolute analyte area was measured in line scan mode for selected antiretroviral drugs, and the results are presented in Table III- 5. The residual standard deviation from four spots was up to 30%, but not significantly matrix dependent. Such variability is typical for MALDI-SRM/MS analyses with dried droplet sample preparation, making the use of an internal standard almost mandatory for quantitative analysis.^{47, 49} The spot-to-spot variability for all 47 analytes may be found in Table S2, the results were similar to those of Table III- 5. The hypothesis above that the more lipophilic NpCCA and AnCCA might be better suited for lipophilic analytes is not supported by these data.

Table III- 5. Spot-to-spot reproducibility for selected antiretroviral drugs. The numbers indicate the absolute area (in counts) given by the SRM trace of the analyte of interest and the RSD is given in parenthesis. The concentration for all analytes was 5 ng/ μ L.

Compound	CHCA	NpCCA	AnCCA	Cl-CCA
Saquinavir	1.89e+07 (20%)	5.10e+07 (31%)	3.96 e+07 (11%)	7.40e+07 (6%)
Indinavir	5.97e+06 (13%)	1.85e+07 (21%)	1.14e+07 (19%)	4.13e+07 (7%)
Nelfinavir	6.68e+06 (13%)	9.11e+06 (18%)	1.54e+07 (15%)	2.76e+07 (18%)
Atazanavir	1.75 e+06 (25%)	4.34e+06 (21%)	3.03e+06 (29%)	4.12e+07 (9%)
Ritonavir	5.97e+05 (14%)	1.56e+06 (12%)	1.97e+05 (28%)	1.17e+07 (12%)
Amprenavir	5.98e+05 (4%)	5.13e+04 (18%)	6.25e+04 (16%)	4.17e+06 (6%)
Lopinavir	8.09e+04 (9%)	2.85e+05 (11%)	7.03e+04 (19%)	1.50e+06 (8%)

c) Matrix absorbance at 337 and 355 nm

During the MALDI ionization process, the laser energy is absorbed by the matrix, leading to its excitation and then its ionization. Thus, a suitable matrix should have a strong absorbance at the working laser wavelength, typically 337 nm (N_2 laser) or 355 nm (frequency tripled Nd:YAG). Although the solution spectra help to characterize the matrix substances, the MALDI sample is in the solid state. Therefore, UV absorption spectra were acquired directly from powdered matrices. As seen in Figure III- 8, the three newer matrices all absorb at longer wavelengths than CHCA. As expected, increasing the size of the aromatic ring system shifts the lowest energy absorption to the red. Also as expected, solid state intermolecular interactions lead to considerable band broadening, reducing the degree of peak to valley modulation in the spectra, compared to the solution phase spectra in Figure III- 6. As shown in the figure, at both 337 and 355 nm all these matrices have rather similar absorbance and are quite suitable for MALDI.

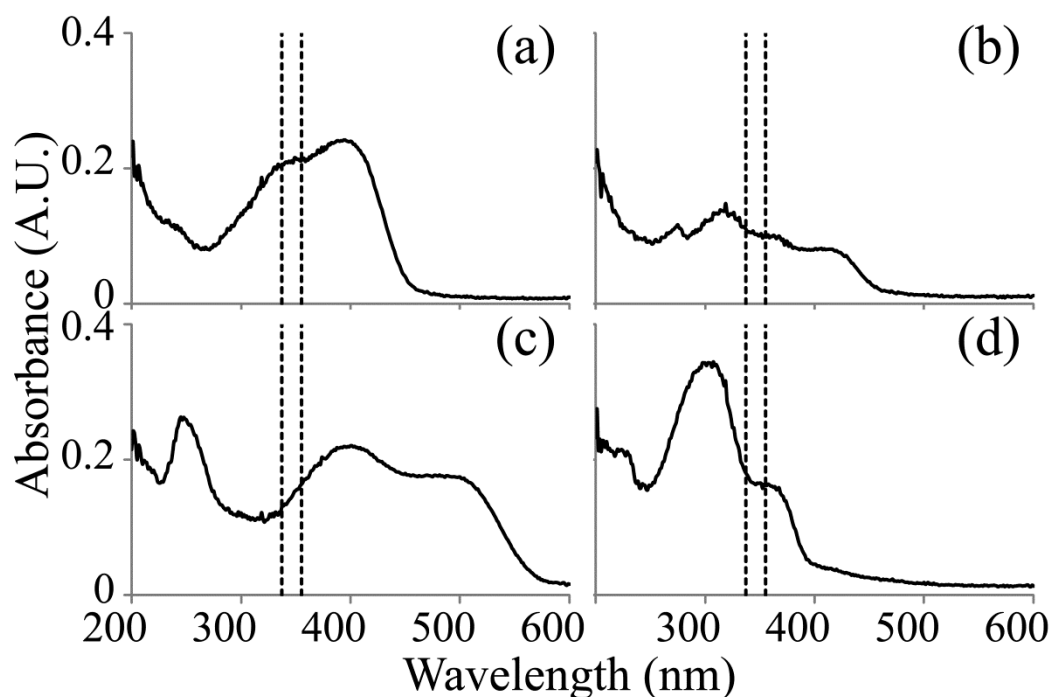


Figure III- 8. Solid-state UV absorption spectra of the matrices: (a) CHCA; (b) NpCCA; (c) AnCCA and (d) Cl-CCA. The laser wavelengths typically used in MALDI, 337 nm and 355 nm, are marked with vertical dotted lines.

d) Full scan quadrupole MS spectra of the matrices

A major motivation for synthesizing new matrices was to move interfering matrix-derived background ions to different m/z regions than for CHCA. Figure III- 9 shows the MALDI matrix spectra obtained in positive ionization mode using a triple quadrupole analyzer fitted with a Nd:YAG laser (355 nm) and operated in single quadrupole mode (Q1 mode). In each case, the laser energy applied was slightly above the ionization threshold.

The MALDI-Q spectrum of CHCA shows predominant molecular peaks at m/z 172, 190, 212, and 228, corresponding to the fragments and adducts commonly observed for this matrix in positive ionization mode. CHCA also presents an intense peak at m/z 379 corresponding to the $[2M+H]^+$ cluster ion, and also several ions assigned to adducts and fragments of the non-covalent trimer. The same general pattern is observed for the chlorinated matrix Cl-CCA (Figure III- 9.d), including trimer ions. Both NpCCA and AnCCA are generally similar to the other two, but with less trimer signal. Since the patterns are broadly similar in terms of the types of ions observed, they are shifted by the respective differences in molecular weight (18 u for Cl-CCA, 34 u for NpCCA, and 84 u for AnCCA). For NpCCA (Figure III- 9.b), very few background ions are present below m/z 206. For AnCCA (Figure III- 9.c), no ions are found below m/z 230.

As a comparison, Figure III- 10 displays positive MALDI-MS spectra of matrices using a MALDI-TOF analyzer equipped with a N_2 laser (337 nm, 10 Hz), and it's noteworthy that the same patterns are observed, whatever the laser used.

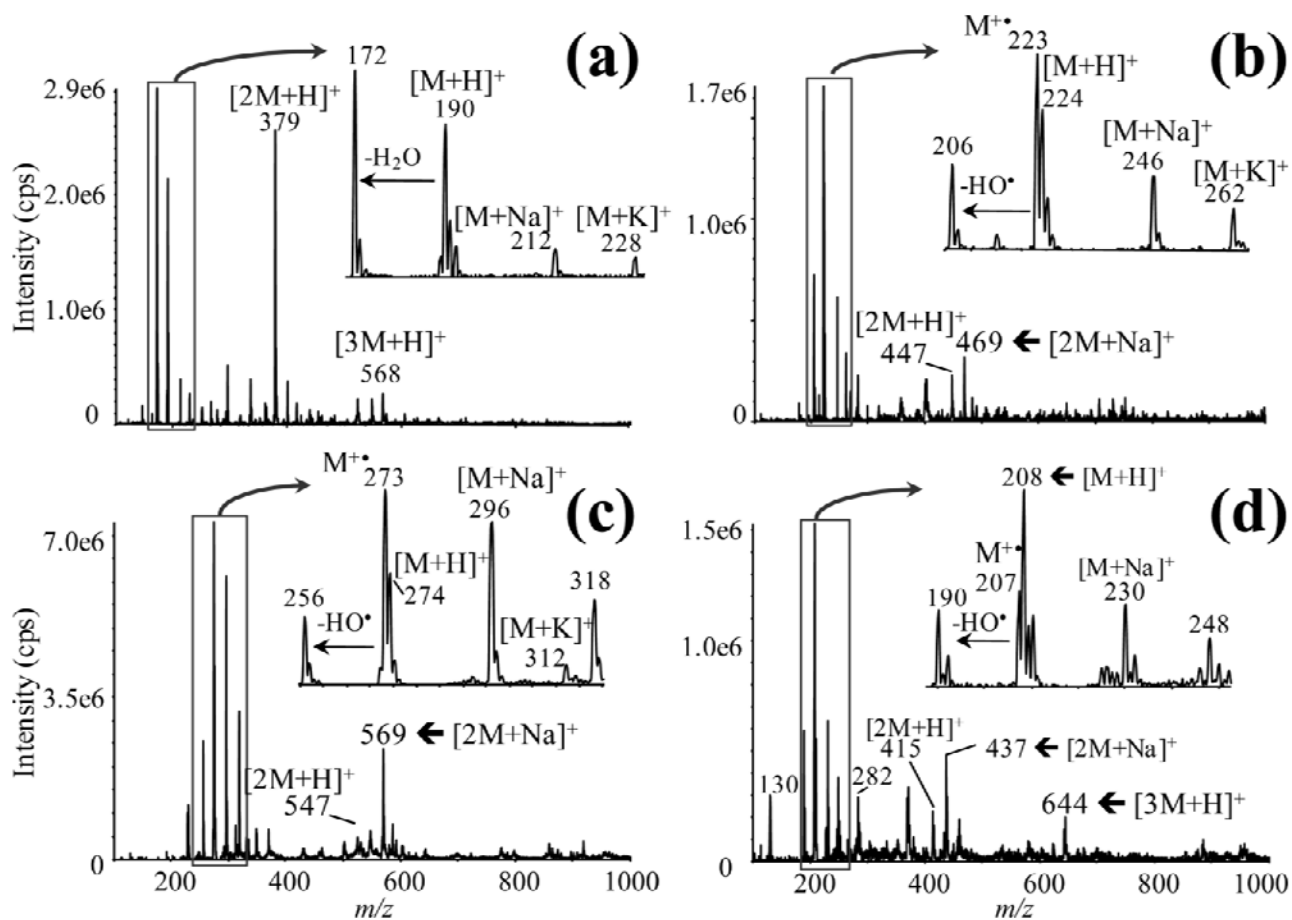


Figure III- 9. Positive MALDI-MS spectra of matrices using a triple quadrupole analyzer operated in Q1 mode: (a) CHCA (1.4 $\mu\text{J}/\text{pulse}$); (b) NpCCA (1.4 $\mu\text{J}/\text{pulse}$); (c) AnCCA (1.7 $\mu\text{J}/\text{pulse}$) and (d) Cl-CCA (1.5 $\mu\text{J}/\text{pulse}$). The molecular ion regions are expanded to show the common fragments and adducts observed.

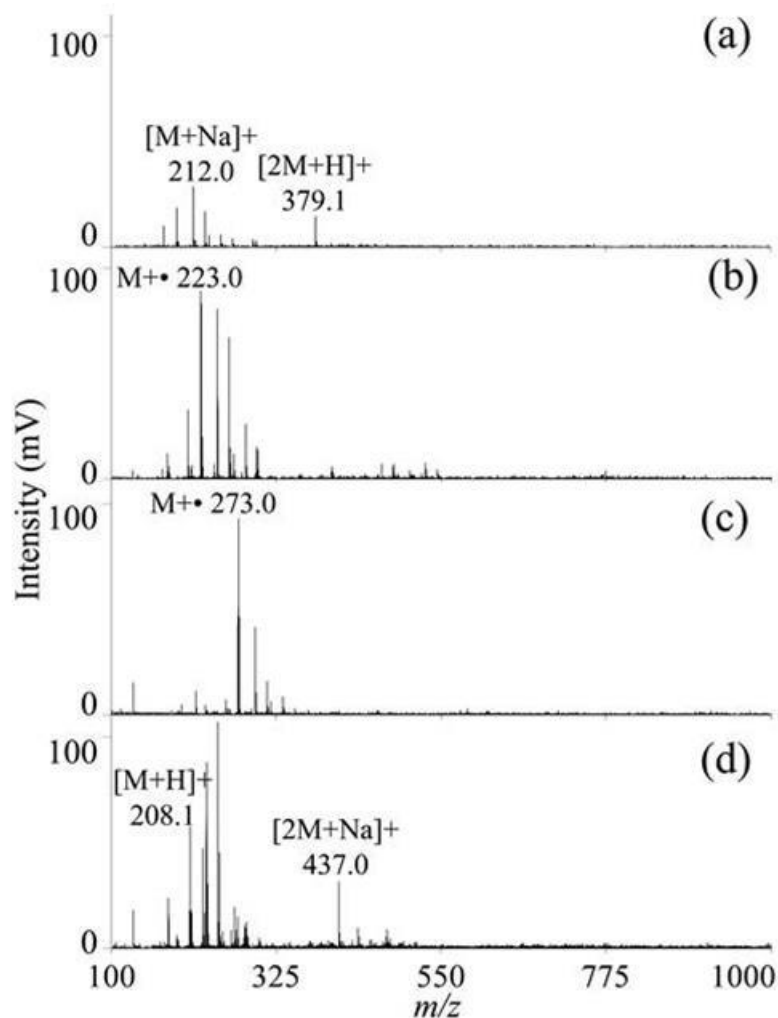


Figure III- 10. Positive MALDI-MS spectra of matrices using a TOF analyzer (laser energy used for each matrix, arbitrary unit): (a) CHCA (90); (b) NpCCA (95); (c) AnCCA (120) and (d) Cl-CCA (100). MALDI-TOF spectra were acquired on a Axima-CFR+ MALDI TOF mass spectrometer (Kratos Analytical, Manchester, UK) using a N2 laser (337 nm, 10 Hz).

3.2.4.2. Experimental determination of matrix proton affinities

Based on current models of MALDI ionization process, where primary ions of the matrix are generated by the laser desorption/ionization followed by proton transfer reactions to ionize analyte,⁷ several studies have investigated the correlation between the gas phase proton affinity (PA) of the matrix and the formation of protonated analyte ions.⁶⁸ These studies suggest that a matrix possessing a lower proton affinity is more capable of transferring a proton to the analyte,^{71, 75} enabling the protonation of a wider range of compounds. Therefore, the proton affinities of the synthesized matrices were determined by Cooks' kinetic method.⁶⁷ The PA of each matrix was determined within a confidence interval of 95% for 36 measurements at collision energies ranging from 5 to 8.5 eV, with a step of 0.1 eV. This interval was chosen so that the molecular ions do not fragment at such collision energies, as shown Figure III- 11.a,c, where the signal for CHCA and Cl-CCA are monitored. The signal decrease for the protonated ion $[\text{CHCA}+\text{H}]^+$ m/z 190 starts at *ca.* CE=8.5 eV and corresponds to formation of the ion m/z 172 (= -18u compared to $[\text{CHCA}+\text{H}]^+$ which suggests a loss of H_2O from $[\text{CHCA}+\text{H}]^+$). Figure III- 11.b).

To validate the method, the calculation of the proton affinities of CHCA, sinapinic acid and caffeic acid were first performed (i.e. SA and caffeic acid were chosen because they are both cinnamic acid derivatives). The mean proton affinity value is of 841 ± 0.4 kJ/mol (IC95%) for CHCA (Figure III- 12.a), leading to a precision of 0.1% and an accuracy of 100.0%, in comparison with the reference value of 841 kJ/mol⁷¹⁻⁷² and an accuracy of 110% compared to 766.2 kJ/mol.⁷³ The proton affinity of SA and caffeic Acid were determined to be 883.1 ± 0.1 kJ/mol (Figure III- 12.b) and 868.1 ± 0.2 kJ/mol (Figure III- 12.c), respectively. These results are consistent with the literature, i.e. SA = 887 kJ/mol (accuracy of 99.5%) and caffeic acid = 866 kJ/mol (accuracy = 100.2%).⁷¹

The calculated PA value of 841 kJ/mol for CHCA was used for the determination of the proton affinities of the synthesized matrices. The Cooks plots obtained are given Figure III- 12 and the resulting proton affinities values are: $\text{PA}_{\text{Cl-CCA}} = 815.5 \pm 0.3$ kJ/mol (Figure III- 12.d), $\text{PA}_{\text{NpCCA}} = 837.3 \pm 0.1$ kJ/mol (Figure III- 12.e) and $\text{PA}_{\text{AnCCA}} = 856.8 \pm 0.2$ kJ/mol (Figure III- 12.f). These do not take into account the uncertainties of the reference base PAs. The PA determined for the chlorinated matrix is between the value calculated by *ab initio* methods, 789.9 kJ/mol²⁹ and its previously measured value of 832 kJ/mol.¹³ Nevertheless, the chlorinated derivative still has the lowest proton affinity among these matrices, and is expected to give the best protonation efficiency.

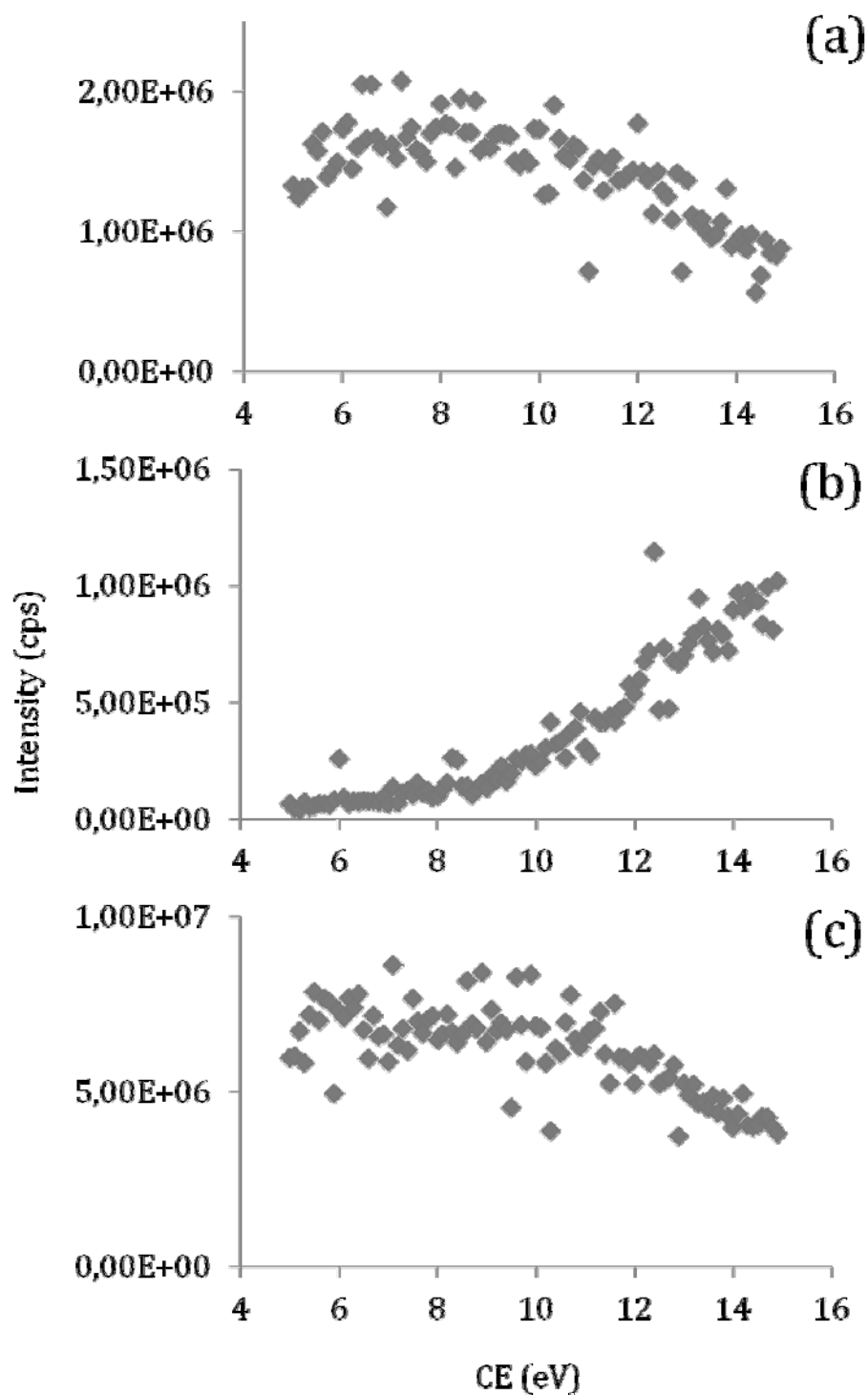


Figure III- 11. Evolution of the absolute signal measured for (a) $[\text{CHCA}+\text{H}]^+$ at m/z 190; (b) $[\text{CHCA}-\text{H}_2\text{O}+\text{H}]^+$ at m/z 172 and (c) $[\text{Cl}-\text{CCA}+\text{H}]^+$ at m/z 208, resulting in the fragmentation of the dimer formed between CHCA and Cl-CCA, in function of the collision energy ranging from 5 to 15 eV.

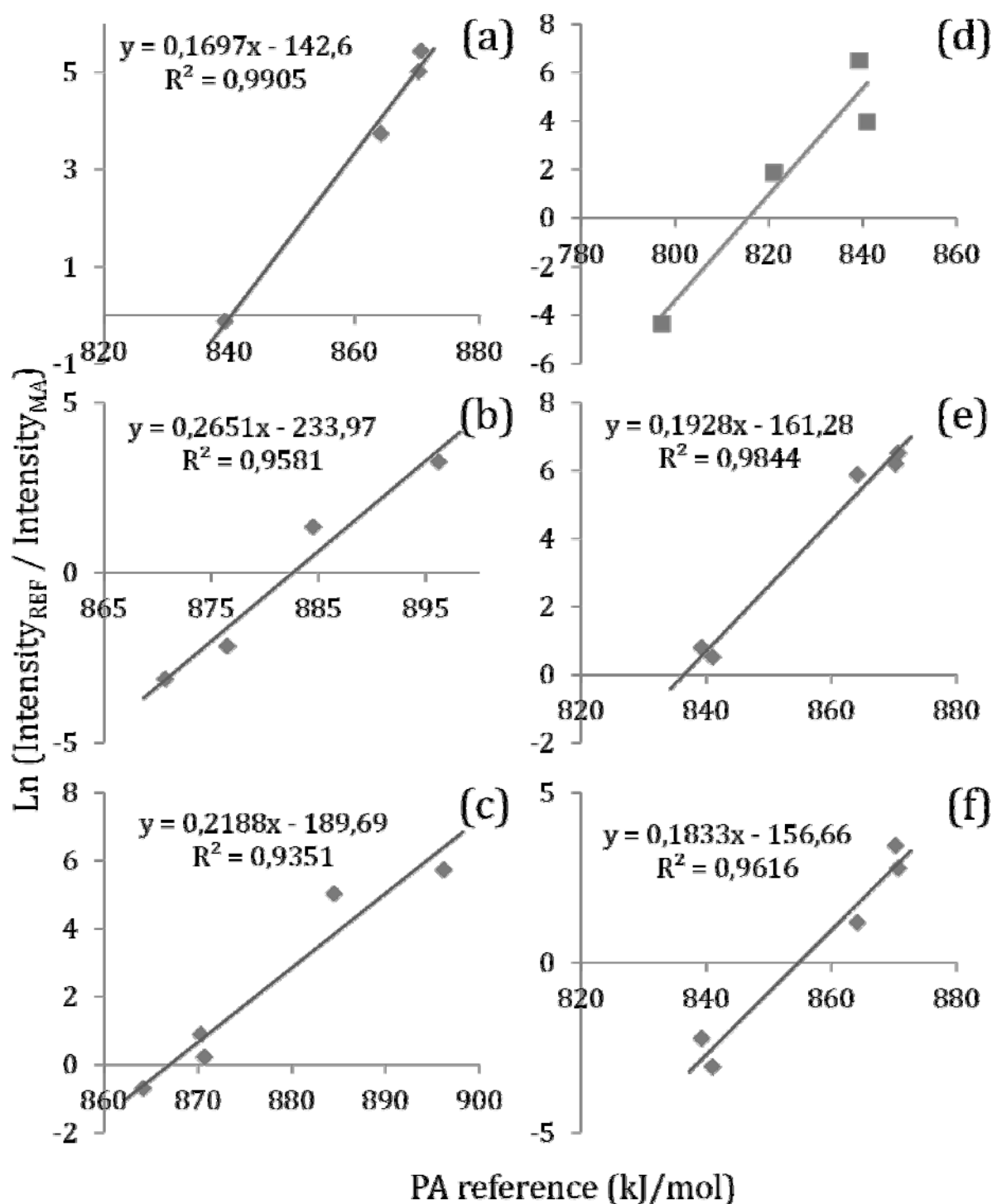


Figure III- 12. Cook plots for: (a) CHCA; (b) SA; (c) caffeic acid; (d) Cl-CCA; (e) NpCCA and (f) AnCCA.

3.2.4.3. Evaluation of the performance of the CHCA-based matrices for SRM analysis of low molecular weight compounds

To characterize the performance of the different matrices in SRM mode, the following factors were investigated i) influence of the laser energy on the analyte signal, ii) effects of interfering matrix ions, iii) comparison of sensitivity for a range of analytes.

a) Influence of the laser energy on the absolute signal and on the S/N ratio of LMWC

Since laser-related parameters, especially the fluence, strongly influence total signal,⁷⁶⁻⁷⁷ the fluence was optimized in order to get the best signal-to-noise ratio for each matrix-analyte pair. As seen in Figure III- 13, for the SRM signal of the antiretroviral saquinavir, below an apparent ionization threshold, no ions are detected.⁷⁸

This apparent threshold was quite similar for many matrix-analyte combinations (around 1.5 μJ , corresponding to approximately 2.4 mJ/cm^2). The maximum detected S/N was generally observed slightly above this threshold. At higher pulse energies, a gradual decrease was generally observed. While higher pulse energy and fluence is expected to increase the number of ions created, it also changes the plume characteristics.³⁷ This may have significant consequences for the ion collection efficiency of the instrument. The decrease in S/N at higher fluence is believed to be largely due to reduced ion transmission, but this was not investigated further. From the data in Figure III- 13, the optimum laser energy for SQV was found to be 1.7 μJ (2.7 mJ/cm^2) for CHCA and NpCCA, and 2.1 μJ (3.3 mJ/cm^2) for AnCCA and Cl-CCA. A similar procedure was used for all other analyte-matrix pairs.

From these individual results, an average energy value was calculated for each matrix. These values are expected to be appropriate for general analyte mixtures. The pulse energies used for the results presented below were then: $2.0 \pm 0.4 \mu\text{J}$ (3.2 mJ/cm^2) for CHCA; $2.1 \pm 0.2 \mu\text{J}$ (3.3 mJ/cm^2) for NpCCA; $3.0 \pm 0.3 \mu\text{J}$ (4.8 mJ/cm^2) for AnCCA; and $3.6 \pm 0.3 \mu\text{J}$ (5.7 mJ/cm^2) for Cl-CCA (95% confidence intervals calculated from 40 measurements).

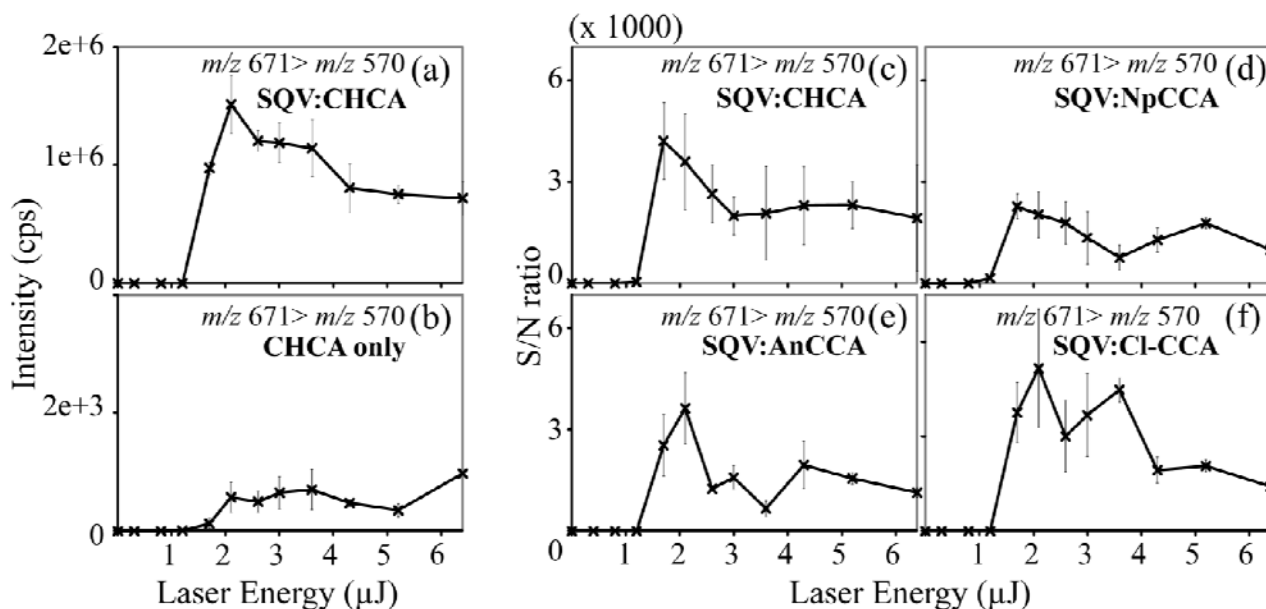


Figure III- 13. Effect of the laser energy on the SRM signal of saquinavir (SQV) for the different matrices. The SQV solution (5 $\text{ng}/\mu\text{L}$) was mixed 1:1 with each saturated matrix solution. (a) SRM transition intensity of SQV at several laser energies from spots of CHCA mixed with SQV and (b) CHCA without SQV (i.e. measure of the noise). S/N ratios at several laser energies using (c) CHCA; (d) NpCCA; (e) AnCCA and (f) Cl-CCA as matrices.

b) Matrix tuning with CHCA derivatives

We are now in a position to select one of the four CCA matrices which interferes least with a given analyte. As an example, consider 2-arachidonyl glycerol (2-AG). Figure 5 shows a line scan of the 2-AG SRM signal across spots of each matrix. The protonated dimer ion of CHCA interferes with the 2-AG protonated molecular ion at m/z 379. However, due to the selectivity of the SRM mode the noise level for CHCA is relatively low (< 100 cps) and a S/N ratio of 13 at a concentration of 15 ng/ μ L (Figure III- 14.a,e) was measured. The absolute signal is similar in NpCCA, but the lower background (in comparison with the background generated by CHCA) results in a higher S/N of 61. Cl-CCA, which possesses the lower proton affinity, resulted in the highest sensitivity. But background ions are also generated and a high variability from hot spots was observed, leading to a comparable S/N (i.e. 73) than NpCCA. AnCCA appears to be inappropriate for the analysis of 2-AG (S/N < 1 , Figure III- 14.g) because the analyte signal is too weak. These observations are consistent with a secondary proton transfer ionization model, as the absolute signal of 2-AG increases when the PA of the matrices decreases (i.e. AnCCA $>$ CHCA $>$ NpCCA $>$ Cl-CCA).

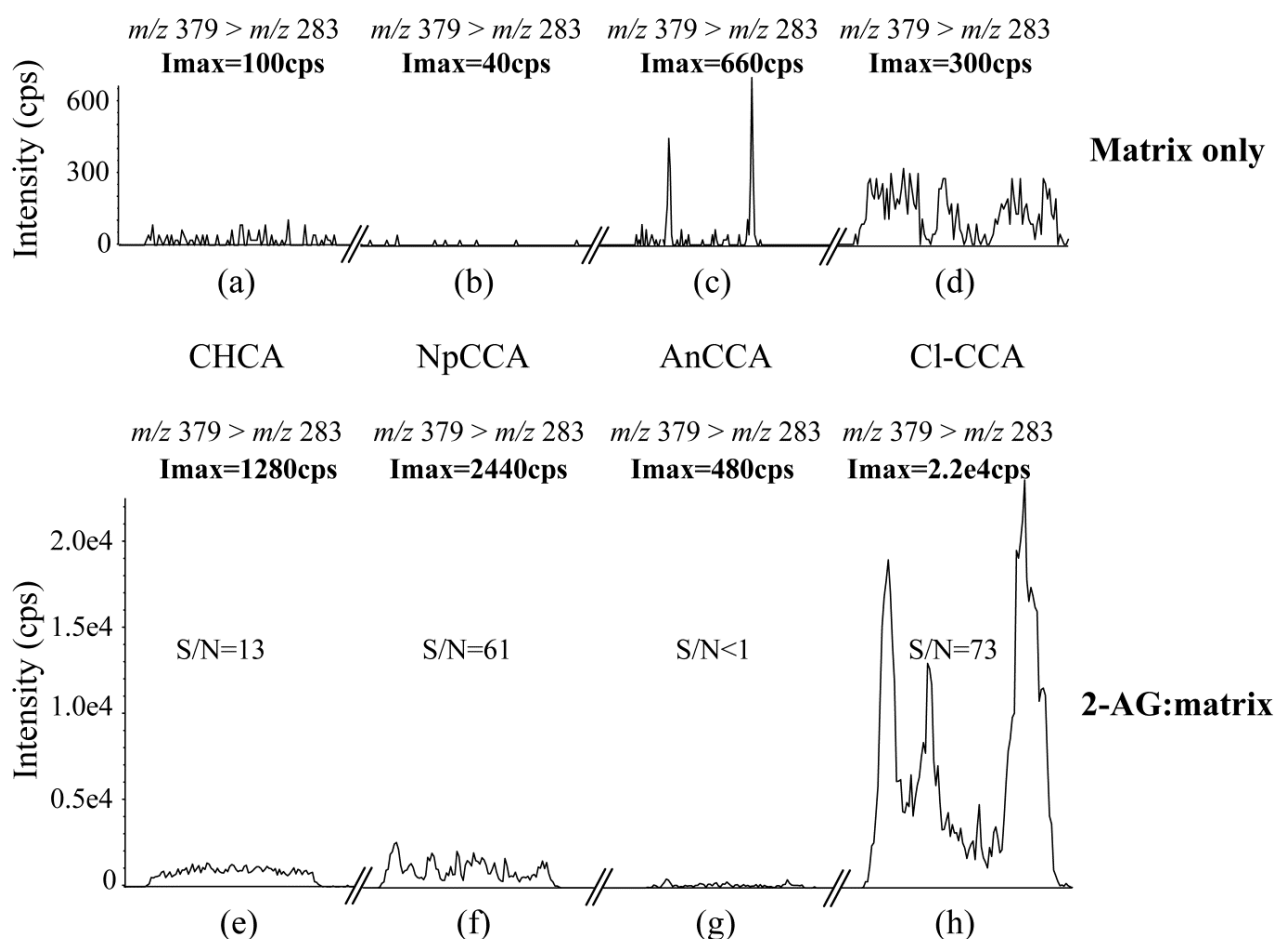


Figure III- 14. Line scan SRM traces of 2-arachidonyl-glycerol from spots containing matrices only (a-d) and from spots containing the analyte (15 ng/ μ L) mixed 1:1 with each matrix (e-h). (a,e) CHCA; (b,f) NpCCA; (c,g) AnCCA and (d,h) Cl-CCA. Time is shown on the abscissa and the width of each window is 0.10 min. The plate speed was 0.5 mm/s.

c) Relative matrix sensitivities in UV-MALDI-SRM analysis of LMWC

Analytical sensitivity was experimentally determined using selected reaction monitoring detection mode with a high repetition rate laser.³⁷ The signal-to-noise ratios (S/N) of a set of LMWC were estimated by comparing SRM signal intensity from identical samples with or without the analyte. See Table III- 2 for the SRM transitions monitored. Table III- 6 gives an overview of the results for all the analytes in the different matrices. Only S/N differences greater than 30 % were considered significant (see also Table III- 6 and Table S2).

The results presented in Table III- 6 illustrate that NpCCA, AnCCA and Cl-CCA are useful alternatives to CHCA for most of the compounds tested. The major benefit of the new matrices is the ability to tune the background ions for a specific analyte. Certain compounds, particularly the antiretrovirals (lopinavir (LPV), amprenavir (APV), ritonavir (RTV), atazanavir (ATV), nelfinavir (NFV), indinavir (IDV), saquinavir (SQV)), as well as fipexide, fluphenazine, risperidone and its hydroxylated metabolite, all showed a sensitivity improvement (*e.g.* up to 80 times better S/N for lopinavir using the chlorinated matrix) with NpCCA, AnCCA and Cl-CCA compared to CHCA (Table III- 6). For the antiretroviral drugs, the S/N ratios based on the SRM transition intensity is systematically improved using the chlorinated matrix compared to the other matrices (Figure III- 15). One such exception is indinavir, for which the noise is 5-times higher with Cl-CCA than with CHCA even though the absolute signal is better (results not shown). Then, there is no S/N enhancement.

Overall, the best results are obtained with the chlorinated matrix, showing relative improvement vs. CHCA for more than half of the compounds. NpCCA and AnCCA are better than with CHCA for at least one third of the 47 compounds. These results are consistent with a lower matrix PA favoring analyte protonation, leading to stronger signal for a wider range of compounds.

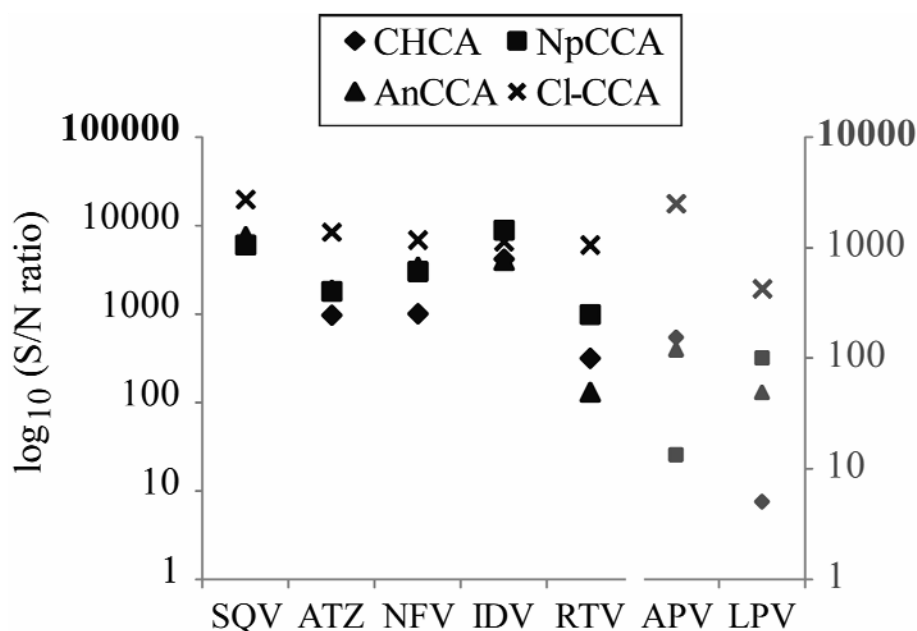


Figure III- 15. Resulting S/N ratios for the MALDI-SRM analysis of seven antiretroviral compounds. The logarithmic scale is only used to clarify the presentation of the data. SQV: saquinavir; ATZ: atazanavir; NFV: nelfinavir; IDV: indinavir; RTV: ritonavir; APV: amprenavir; LPV: lopinavir. Values for APV and LPV are given by the right vertical axis.

3.2.5. Conclusions

Two new MALDI matrices, NpCCA and AnCCA have been synthesized and characterized as alternatives to the recently reported Cl-CCA, and the common matrix CHCA for quantitative MALDI-SRM analysis of low molecular weight compounds. Matrix-related background ions were observed at higher m/z values for AnCCA, NpCCA and Cl-CCA compared to CHCA, while retaining considerable chemical similarity to that matrix. By selecting an alternative matrix it may be possible to improve the S/N for many analytes in SRM mode, while the situation may be different in full scan. Compared to CHCA, Cl-CCA, yielded an improved sensitivity (at least 30% increase in S/N) for 60% of the LMWC tested, while NpCCA and AnCCA enabled a better S/N for more than 30% of the compounds. The results appear to be generally consistent with secondary charge transfer reactions of matrix with analyte. The matrix with the lowest PA, Cl-CCA generally gave the highest absolute signal, with the intensity trends following the proton affinities. Further work is in progress to evaluate these matrices for analysis of biological extracts, where endogenous compounds may compete with the analytes for ionization.

Table III- 6. Analyte S/N comparison for the four different matrices. Analytes concentration is 5 ng/ μ L.

Analyte	NpCCA	AnCCA	CI-CCA
17-OH-Progesterone	-	-	+++
2-Arachidonyl-Glycerol	+++	n.d.	+++
α -Lactose*	n.d.	n.d.	n.d.
Adenine	-	---	-
Aldosterone*	n.d.	+++	+++
Amprenavir	---	0	+++++
Atazanavir	++	++	++++
ATP	---	n.d.	---
Chlorprothixene	+++	++++	+++++
Clonazepam	+++	++	+++
Creatine	--	---	---
Creatinine	--	-	-
Cytidine	---	---	+++
Deoxyuridine	---	---	0
Estrone*	n.d.	n.d.	++++
Everolimus	0	+++	++++
Fipexide	++	++	++++
Fluoxetine	+++++	++++	++++
Fluphenazine	0	0	-
GPCho	---	---	---
Guanosine	--	---	0
Hydrocortisone*	n.d.	n.d.	n.d.
Indinavir	+++	0	++
L-Cysteine	++++	++	++++
L-Glutathione	---	---	++
L-Phenylalanine	-	--	++
L-Proline	-	---	0
L-Tryptophan	--	0	0
L-Tyrosine	n.d.	---	---
Levodopa	n.d.	--	+
Lopinavir	+++++	++++	+++++
Methaqualone	---	---	---
Nelfinavir	+++	+++	++++
Nordiazepam	---	---	--
Norfluoxetine	+++++	+++	++++
Nortriptyline	+++	+++	+++
Propranolol	+	-	0
Reserpine	+	++++	---
Risperidone	0	--	-
Risperidone-OH	-	++	---
Ritonavir	+++	--	+++++
Saquinavir	-	0	+++

Table III-6. Continued.

Analyte	NpCCA	AnCCA	Cl-CCA
Talinolol	0	--	+++
Testosterone	--	--	+++
Thymine	0	n.d.	++
Uracil	0	---	+++
Venlafaxine	+++	++++	+++

* analyte not detected ($S/N < 3$) using CHCA as matrix

n.d. not detected ($S/N < 3$)

--- $S/N_{(A:M)} < 0.3 \times S/N_{(A:CHCA)}$

-- $0.3 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 0.5 \times S/N_{(A:CHCA)}$

- $0.5 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 0.7 \times S/N_{(A:CHCA)}$

0 $0.7 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 1.3 \times S/N_{(A:CHCA)}$

+ $1.3 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 1.5 \times S/N_{(A:CHCA)}$

++ $1.5 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 2.0 \times S/N_{(A:CHCA)}$

+++ $2.0 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 5.0 \times S/N_{(A:CHCA)}$

++++ $5.0 \times S/N_{(A:CHCA)} < S/N_{(A:M)} < 10 \times S/N_{(A:CHCA)}$

+++++ $10 \times S/N_{(A:CHCA)} < S/N_{(A:M)}$

(A = analyte, M = NpCCA, AnCCA or Cl-CCA)

3.2.6. Supplemental material

Table S1. Analyte physicochemical properties. MA: most acidic pKa. MB: most basic pKa. (n.a. = not available). ^anucleoside. ^bsteroid. ^csterol (lipid). ^dphospholipid. ^eisoquinoline. ^famino acid. ^gamino alcohol. ^hpyrimidine. ⁱpyridine. ^jnucleotide. ^kpiperazine. ^lphenothiazine. ^mbenzodiazepine. ⁿindole alkaloid. ^othiazole. ^ppyrimidinone. ^qquinazolinone. ^rpurine. Antiretroviral drugs are underlined. Values were calculated at 25°C.

Compound name	MW (g/mol)	pKa MA / MB	cLogP	cLogD (pH3)	cLogD (pH8)
Deoxyuridine ^a	228.20	8.39±0.20 / n.a.	-1.70±0.49	-1.70	-1.85
Aldosterone ^b	360.44	12.95±0.10 / n.a.	0.73±0.45	0.72	0.72
Testosterone ^b	288.42	15.06±0.60 / n.a.	3.48±0.23	3.47	3.47
Estrone ^b	270.37	10.25±0.40 / n.a.	3.69±0.30	3.69	3.69
17-OH-Progest. ^b	330.46	13.03±0.60 / n.a.	2.89±0.36	2.89	2.89
Hydrocortisone ^c	362.46	12.47±0.70 / n.a.	1.43±0.47	1.43	1.43
GPCho ^d	762.20	1.23±0.50 / n.a.	11.94±0.63	12.53	12.54
2-AG	378.55	13.54±0.10 / n.a.	6.25±0.75	6.24	6.24
α-lactose	342.30	12.02±0.70 / n.a.	-3.41±0.49	-3.41	-3.41
<u>Saquinavir</u> ^e	670.80	11.05±0.46 / 7.61±0.40	4.44±0.86	1.34	3.74
Pro ^f	115.13	2.35±0.20 / 10.70±0.20	-0.57±0.28	-3.13	-3.07
Trp ^f	204.23	2.30±0.10 / 10.31±0.13	1.04±0.31	-1.52	-1.46
Phe ^f	165.19	2.21±0.10 / 9.20±0.15	1.11±0.29	-1.43	-1.41
Tyr ^f	181.19	2.25±0.10 / 9.35±0.15	0.38±0.30	-2.17	-2.15
Cys ^f	121.16	2.07±0.10 / 11.05±0.16	0.24±0.39	-2.30	-2.61
Gly ^f	307.32	2.21±0.10 / 9.30±0.16	-0.87±0.74	-3.49	-4.41
Creatine ^f	131.13	3.43±0.10 / 13.51±0.70	-1.88±0.64	-3.97	-4.38
Levodopa ^f	197.19	2.24±0.20 / 9.30±0.30	-0.23±0.32	-2.77	-2.76
Propranolol ^g	259.34	13.84±0.20 / 9.14±0.38	3.10±0.25	0.00	1.00
Uracil ^h	112.09	9.20±0.21 / -4.19±0.20	-0.71±0.29	-0.71	-0.71
Thymine ^h	126.11	9.64±0.40 / -4.15±0.40	-0.12±0.29	-0.12	-0.13
<u>Indinavir</u> ⁱ	613.79	14.40±0.40 / 5.19±0.10	2.88±0.86	-0.65	2.86

Table S1. Continued.

Compound name	MW (g/mol)	pKa MA / MB	cLogP	cLogD (pH3)	cLogD (pH8)
Cytidine ^a	243.22	13.48±0.70 / 3.27±0.70	-1.94±0.45	-2.38	-1.93
Guanosine ^a	283.24	9.23±0.20 / 1.69±0.50	-1.72±0.57	-1.76	-1.74
ATP ⁱ	507.18	0.80±0.50 / 3.40±0.50	-4.42±0.86	-8.45	-10.46
Fluphenazine ^l	437.52	14.96±0.10 / 6.85±0.70	4.84±0.46	1.38	4.80
Nordiazepam ^m	270.71	11.72±0.70 / 3.40±0.10	3.15±0.47	2.62	3.15
Clonazepam ^m	315.71	11.21±0.70 / 1.55±0.25	2.34±0.56	2.32	2.34
Reserpine ⁿ	608.68	18.11±0.70 / 7.25±0.70	4.05±0.71	0.98	3.60
<u>Nelfinavir</u> ^e	567.78	9.58±0.10 / 6.21±0.40	7.28±0.77	4.65	7.28
<u>Ritonavir</u> ^o	720.94	11.47±0.46 / 3.48±0.50	5.28±0.89	4.68	5.28
<u>Amprenavir</u>	505.63	11.54±0.46 / 1.76±0.10	4.20±0.51	4.18	4.20
Everolimus	958.22	10.40±0.70 / -2.15±0.70	3.39±0.88	3.38	3.38
Talinolol	363.49	13.82±0.20 / 9.16±0.48	3.20±0.36	0.10	1.08
Venlafaxine	277.40	14.84±0.20 / 9.26±0.28	2.91±0.35	-0.19	0.70
<u>Atazanavir</u>	704.86	11.11±0.46 / 4.65±0.25	5.20±0.74	4.47	5.20
<u>Lopinavir</u>	628.80	13.89±0.46 / -0.75±0.20	6.26±0.84	6.26	6.26
Risperidone-OH ^p	426.48	13.00±0.60 / 7.86±0.40	1.52±0.43	-1.69	1.28
Risperidone ^p	410.48	n.a. / 7.89±0.40	2.89±0.41	-0.57	2.63
Methaqualone ^q	250.30	n.a. / 2.92±0.70	2.50±0.41	2.24	2.50
Fluoxetine ^k	309.33	n.a. / 10.05±0.10	4.09±0.45	0.99	1.31
Fipexide ^k	388.84	n.a. / 5.38±0.70	3.05±0.63	0.74	3.04
Adenine ^r	135.13	n.a. / 10.43±0.20	-2.21±1.00	-4.12	-3.99
Creatinine ^f	113.13	n.a. / 6.89±0.20	-1.83±0.62	-3.82	-1.89
Chloroprothixène	315.86	n.a. / 9.05±0.28	6.05±1.06	2.95	4.03
Nortriptyline	263.38	n.a. / 10.00±0.10	5.65±0.32	2.55	2.90
Norfluoxetine ^k	295.30	n.a. / 9.05±0.13	4.36±0.42	1.26	2.34

Table S2. Area measured from SRM trace of each analyte in the four matrices and the corresponding RSD (n=4). * RSD values in red correspond to spot-to-spot variability above 30%.

Matrix Compound	CHCA		NpCCA		AnCCA		Cl-CCA	
	Area (counts)	RSD* (%)	Area (counts)	RSD* (%)	Area (counts)	RSD* (%)	Area (counts)	RSD (%)
Adenine	1.72E+07	8%	1.03E+07	16%	6.18E+06	8%	3.28E+07	16%
Aldosterone	1.12E+06	13%	2.42E+05	13%	5.10E+04	8%	5.86E+05	6%
Amprenavir	5.98E+05	4%	5.13E+04	18%	6.25E+04	16%	4.17E+06	6%
2-Arachidonyl Glycerol	2.18E+04	4%	2.99E+04	24%	3.68E+03	1%	9.32E+04	22%
Atazanavir	1.75E+06	25%	4.34E+06	21%	3.03E+06	29%	4.12E+07	9%
ATP	3.41E+04	16%	6.11E+03	35%	no signal	n.a.	1.17E+04	2%
Chloroprothixene	4.81E+06	19%	1.51E+06	20%	1.01E+07	2%	2.25E+07	6%
Clonazepam	1.17E+06	3%	2.19E+05	19%	6.67E+05	30%	2.71E+06	9%
Creatine	1.90E+07	7%	1.22E+07	13%	8.57E+06	19%	1.79E+07	14%
Creatinine	5.50E+06	24%	9.64E+05	12%	2.13E+06	24%	4.69E+06	13%
L-Cysteine	1.07E+05	24%	4.69E+04	29%	5.09E+03	19%	1.00E+05	19%
Cytidine	7.93E+05	7%	1.52E+05	35%	2.20E+05	6%	4.50E+06	19%
Deoxyuridine	7.19E+04	19%	4.14E+04	18%	8.54E+03	4%	2.10E+05	4%
Estrone	6.36E+04	11%	1.72E+04	7%	2.33E+04	21%	4.52E+05	18%
Everolimus	1.07E+06	9%	3.66E+06	21%	6.88E+06	13%	5.56E+06	24%
Fipexide	1.51E+06	15%	1.23E+07	21%	3.42E+06	31%	6.87E+07	8%
Fluoxetine	9.79E+04	17%	7.21E+05	19%	3.48E+05	8%	2.05E+06	17%
Fluphenazine	2.10E+07	18%	3.90E+07	22%	3.54E+07	20%	7.45E+07	21%
L-Glutathione	9.87E+04	12%	3.86E+04	24%	7.55E+03	14%	3.01E+05	21%
GPCho	7.22E+05	16%	4.35E+05	30%	3.20E+05	25%	5.01E+05	10%
Guanosine	2.64E+06	16%	1.24E+06	22%	1.62E+05	17%	1.93E+06	3%
Hydrocortisone	3.28E+04	7%	2.38E+04	10%	3.88E+03	31%	1.86E+04	12%
17-OH-progesterone	2.38E+05	10%	6.36E+04	29%	6.47E+04	17%	8.15E+05	8%
Hydroxy-risperidone	1.32E+08	20%	1.99E+08	4%	1.68E+08	8%	2.71E+08	11%
Indinavir	5.97E+06	13%	1.85E+07	21%	1.14E+07	19%	4.13E+07	7%
Levodopa	1.21E+05	20%	3.89E+05	24%	1.83E+04	17%	2.49E+05	13%
α -Lactose	3.43E+04	11%	9.58E+04	25%	3.13E+04	12%	3.64E+04	14%
Lopinavir	8.09E+04	9%	2.85E+05	11%	7.03E+04	19%	1.50E+06	8%
Methaqualone	1.88E+07	17%	7.43E+06	14%	3.63E+06	27%	5.44E+07	19%
Nelfinavir	6.68E+06	13%	9.11E+06	18%	1.54E+07	15%	2.76E+07	18%
Nordiazepam	5.81E+06	10%	6.86E+06	15%	7.52E+06	19%	1.64E+07	19%
Norfluoxetine	1.34E+04	16%	1.28E+05	17%	1.96E+04	26%	1.72E+05	6%
Nortipyline	3.46E+06	9%	5.97E+06	22%	6.40E+06	19%	7.61E+06	10%
L-Phenylalanine	2.28E+06	13%	3.81E+06	22%	1.07E+06	19%	9.29E+06	10%
L-Proline	7.26E+06	21%	7.16E+06	25%	5.78E+06	10%	1.72E+07	6%
Propranolol	1.24E+07	19%	6.58E+06	15%	8.84E+06	23%	2.00E+07	14%
Reserpine	5.32E+05	13%	3.03E+05	15%	2.28E+06	22%	5.53E+06	11%
Risperidone	1.70E+08	4%	3.24E+08	15%	2.38E+08	6%	3.60E+08	2%
Ritonavir	5.97E+05	14%	1.56E+06	12%	1.97E+05	28%	1.17E+07	12%

Table S2. Continued.

Matrix	CHCA		NpCCA		AnCCA		Cl-CCA	
Compound	Area (counts)	RSD* (%)	Area (counts)	RSD* (%)	Area (counts)	RSD* (%)	Area (counts)	RSD (%)
Saquinavir	1.89E+07	20%	5.10E+07	31%	3.96E+07	11%	7.40E+07	6%
Talinolol	2.58E+05	6%	7.94E+05	37%	5.76E+05	10%	1.06E+06	14%
Testosterone	1.95E+05	8%	3.62E+05	31%	7.07E+04	10%	5.71E+05	10%
Thymine	1.71E+05	10%	2.31E+05	29%	2.77E+03	24%	3.28E+05	20%
L-Tryptophan	5.76E+05	8%	9.30E+04	17%	4.73E+05	16%	1.92E+06	10%
L-Tyrosine	6.15E+05	14%	9.21E+05	12%	1.34E+05	8%	9.98E+05	10%
Uracil	1.36E+05	10%	1.50E+05	19%	6.49E+03	10%	6.26E+05	20%
Venlafaxine	6.77E+06	5%	9.47E+06	19%	1.21E+07	27%	2.23E+07	24%

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 3 - Analysis of tropane alkaloids by MALDI-mass spectrometry imaging in intact *E.coca* leaves



3.3.1. Introduction / Context of the study

This work has been carried out in collaboration with J. Jirschitzka and J.C. D'Auria, from the Department of Biochemistry, Max Planck Institute for Chemical Ecology, Beutenberg Campus, Jena, Germany, involved in the biochemistry and the evolution of tropane alkaloid biosynthesis.⁷⁹

Tropane alkaloids represent a major class of plant-derived secondary metabolites, known to occur in the Solanaceae family, but are also present in the families Convolvulaceae, Proteaceae, Rhizophoraceae and Erythroxylaceae. The diversity of tropane alkaloids is due to different types of modifications of the main core of their structure, and recent work have demonstrated that the plant tropane alkaloid biosynthesis have evolved independently in the Solanaceae and Erythroxylaceae.⁸⁰

The genus *Erythroxylum* (family Erythroxylaceae) contains approximately 230 species with ranges spread throughout the tropics including South America and Madagascar. *Erythroxylum coca* and *Erythroxylum novogranatense* are the most widely used species for the production of cocaine. However, very little is known as to the biological and ecological roles that cocaine and other tropane alkaloids play in plants.

The objectives of the Groups of the Biochemistry Department is to elucidate the the biochemical steps which lead to the production of tropane alkaloids in *Erythroxylum coca* plants.⁷⁹ With this respect, the present work aims at identifying and characterizing cocaine synthase, which is involved in the final step of the biosynthetic pathway leading to the production of cocaine in *E. coca*. Cocaine synthase is capable of producing both cocaine and cinnamoylcocaine *via* the activated benzoyl- or cinnamoyl-CoA thioesters, respectively. Cocaine synthase activity is highest in the young developing leaf tissues and accumulates in the palisade parenchyma and spongy mesophyll.

The growing interest for MS-based molecular imaging approaches in plant biology has already been exposed in the second Chapter of this thesis. Here, matrix-assisted laser desorption/ionization (MALDI) mass spectrometric imaging (MSI), in combination with a triple quadrupole linear ion trap platform, has been used as a qualitative tool to visualize the relative distribution and confirm the identity of tropane alkaloids (i.e. cocaine, cinnamoyl cocaine and ecgonine methyl ester) at the surface of intact leaves from *E.coca* species.

This part only focuses on the MALDI-MSI experimental conditions and the results generated. The full manuscript will be presented in APPENDIX 1.

3.3.2. Materials and Methods

3.3.2.1. Chemicals and reagents

Cocaine (COC), cocaine-d₃ (COC-d₃), cinnamoylcocaine (cinCOC) and ecgonine methyl ester (EME) were provided by Lipomed (Arlesheim, Switzerland). Methanol (MeOH, HPLC grade) was provided by Sigma-Aldrich (Buchs, Switzerland). α -cyano-4-hydroxycinnamic acid (CHCA) was provided by Fluka (Buchs, Switzerland). Formic acid (HCOOH) was purchased from Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

3.3.2.2. Standard and matrix solutions

α -cyano-4-hydroxycinnamic acid CHCA was dissolved at a concentration of 10 mg/mL in MeOH:H₂O:HCOOH (75:25:0.1, v/v/v). Deuterated cocaine (COC-d₃) was added to the matrix solution (final concentration of 200 ng/mL) and sprayed simultaneously with the matrix. Matrix solutions were kept in amber glass bottles to prevent their degradation by UV and stored at 4°C between uses.

3.3.2.3. Sample preparation for the analysis of intact leaves

Leaves were collected from *Erythroxylum coca* (*E. coca*) species at the Botanical Garden of Zurich (Zurich, Switzerland). Samples were collected at three different stages of maturity: stage 1 (referred as “rolled up leaves”); stage 2 (expanding leaves) and stage 3 (mature leaves). After being cut at the basis of the stem, samples were then placed in-between two aluminum foils (to maintain their flat shape), and immersed immediately in liquid nitrogen for flash freezing. Frozen samples were then stored at -80°C prior to the analysis.

After drying in a desiccator under vacuum for ca. 20 minutes at room temperature, intact leaf samples were mounted onto the stainless steel MALDI plate (OPTI TOF 384 well Insert - 123 x 81 mm or OPTI TOF 192 well insert - 44 x 44 mm, AB Sciex, Concord, ON, Canada) using a double-sided adhesive tape (Plasto, Chenove, France). Two intact leaves from the same maturity stage were placed on the MALDI plate in order to analyze both sides of the leaves in the same run. Approximately 12 mL of CHCA 10 mg/ml-solutions were manually sprayed using a Custom Micron CM-C Plus airbrush 0.18 mm (ANEST IWATA Corporation, Yokohama, Japan) held at a distance of ca. 20 cm from the plate. N₂ was used as the nebulizer gas (purity > 99.995%, Messer, Switzerland) and operated at a constant pressure of 0.5 bars.

3.3.2.4. Mass spectrometry imaging

MALDI mass spectrometry imaging acquisitions were performed on a triple quadrupole linear ion trap mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a MALDI source and a frequency-tripled Nd:YAG laser 355 nm (elliptical beam shape of 100x200 μ m). MS and MS/MS images were acquired in positive ionization mode. General operating conditions were: data acquisition mode = line scan in rastering mode, repetition rate laser =

1'000 Hz; laser energy = 60 μ J; MALDI source and q_0 region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q_2 = $2.4 \cdot 10^{-5}$ Torr (nitrogen); declustering potential = 70 V; entrance potential = 10 V; quadrupole resolution was set to unit for Q_1 and Q_3 . Full MS spectra in Q_3 mode were acquired within the range of m/z 50 – 1'050 at a scan rate of 500 u/s and a total scan time (TST) of 2.005 s. The distance between two line scans was of 1 mm and the MALDI plate was moved at a speed of 0.5 mm/s leading to pixels of 1x1 mm using TissueView data processing.

SRM data were acquired by monitoring simultaneously the following transitions: m/z 304 > m/z 182 for COC (CE = 35 eV), m/z 307 > m/z 185 for COC- d_3 (CE = 35 eV), m/z 330 > m/z 182 for cinCOC (CE = 40 eV) and m/z 200 > m/z 182 for EME (CE = 40 eV). The dwell time was set to 500 ms (TST = 2.020 s) and the distance between two line scans was of 1 mm (rastering speed of 0.5 mm/s), resulting in MALDI-SRM/MS-based images with pixels of 1x1 mm for the four compounds. To generate MALDI-SRM/MS images at higher resolution (*i.e.* pixels of 50x50 μ m), the plate speed was kept constant at 0.5 mm/s, the dwell time was decreased to 20 ms, and the distance between 2 line scans was set to 50 μ m (oversampling technique, see results and discussion part).

MS/MS spectra in the enhanced product ion (EPI) scan mode were acquired at a plate speed of 1 mm/s and the distance between two line scans was of 2 mm (pixels of 2x2 mm). The conditions for each compounds were: i) for COC: precursor ion = m/z 304; CE = 35 eV, scan range = m/z 80 – 310, TST = 1.991 s ; ii) for cinCOC: precursor ion = m/z 330; CE = 35 eV, scan range = m/z 80 – 340, TST = 2.051 s; iii) for EME: precursor ion = m/z 200; CE = 45 eV, scan range = m/z 80 – 210, TST = 1.991 s. Reference spectra were acquired from a MALDI spot where 1 μ L of a standard solution with the 3 analytes at a concentration of 100 ng/mL mixed 1:1 (v:v) with CHCA (10 mg/mL) was spotted onto the target plate. Spectra for blank samples and for structural confirmation at the different maturation stages were generated by summing 6 vertical adjacent pixels over selected regions.

3.3.2.5. Software for data acquisition and processing

The MALDI source and its laser were controlled using a custom M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and for data collection. PeakView software (v.1.0.0.3, AB Sciex) was used for raw data processing (.wiff files). A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files (.wiff format) into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex) for MS and MS/MS images generation and processing.

3.3.3. Results and discussion

3.3.3.1. Analytical workflow and experimental considerations

a) Analytical workflow

Direct analysis has been found particularly attractive in many fields of applications since it tends to minimize the sample preparation and keeps intact the sample spatial integrity. MALDI-MSI has been used to map metabolites within plant tissues⁸¹⁻⁸³ as well as to monitor specifically cocaine and related compounds (e.g. single hair sample from human for toxicological screening⁴³), and is envisaged to study the localization of storage sites of tropane alkaloids such as cocaine (COC), cinnamoylcocaine (cinCOC) and ecgonine methyl ester (EME).

The sample preparation and associated analytical workflow are quite straightforward for performing MALDI-MS(/MS)-based imaging experiments (Figure III- 16.a). The first step consists in mounting the intact leaf samples onto an adhesive foil preliminary fixed on the stainless steel MALDI plate. A MALDI matrix, used to promote the analyte's ions formation by a secondary proton transfer reaction from primary matrix ions, is then applied over the whole surface of the sample. This sample preparation step is by far the most critical step since the matrix has to be homogeneously applied and the resulting matrix crystals should be as small as possible to prevent the delocalization of the molecules within the samples.⁸⁴ To overcome inhomogeneous matrix coating, the use of a reference compound is preferred. This reference compound is generally a deuterated analogue of the analyte of interest and can be sprayed at the same time than the MALDI matrix.² In our experimental setup, COC-d₃ was used for image normalization and was dissolved in the matrix solution for monitoring homogeneous matrix deposition.

The MS image acquisitions are then performed on a MALDI hybrid triple quadrupole linear ion trap mass spectrometer (QqQ_{LIT}) equipped with a high repetition rate laser (i.e. 1 kHz) necessary for performing MS/MS-like experiments where a quasi-continuous beam of ions is mandatory for high quality data and high speed acquisition (e.g. selected reaction monitoring MS/MS experiments). Among the multiple operating modes available with the QqQ_{LIT} mass analyzer, three types of acquisition were used in this study:

- A full MS acquisition in positive ionization mode was performed to perform untargeted profiling of the leaves' surface over a mass range of m/z 1'000 (both sides were analyzed);
- A selected reaction monitoring (SRM) mode was used for targeted analysis as well as to produce high resolution mass spectrometric images (fast MS duty cycle).
- An enhanced product ion (EPI) scan was used to generate MS/MS spectra of targeted compounds in order to confirm their identity within the samples compared to authentic standards.

The MALDI plate was moved at different speeds and data were acquired in line scan mode (also referred as rastering mode). One of the advantages of MALDI is the partial sample consumption by one path of the laser due to the focused beam that hits the surface of the

sample. It results that multiple acquisitions per samples can be performed. The design for performing multiple acquisitions on a single sample and generating multiple images based on different scan types is described hereafter. The resulted MS and MS/MS images are then processed using TissueView software.

b) Generation of MALDI-MS(/MS) images

Definition of the pixels size and image resolution. The resolution of an MS image, independently of the acquisition type, is defined by its pixels size in X- and Y-axes dimensions (Figure III- 16.b). The pixels dimension along the X-axis is a function of the duty cycle of the MS experiment (or total scan time, TST) combined with the sample plate speed. As an example, to acquire pixels of 1x1 mm and considering that the MALDI plate is moved at 0.5 mm/s, the MS duty cycle has to be adjusted to 2 seconds. To achieve the same X-axis resolution in the SRM mode, the dwell time has to be set to 500 ms for each of the 4 transitions to get a the total scan time (TST) of *ca.* 2 seconds (Figure III- 16.b-ii). Along the same line, to generate images with 50 μm pixels and keeping the same plate speed, the dwell time has to be decreased down to 25 ms including a pause time of 5 ms between each SRM transition (total scan time of 100 ms, Figure III- 16.b-iii).

The Y-axis dimension refers to the distance between two raster lines acquired with the same MS(/MS) experiment type. The Y-axis resolution is limited by the stage motor resolution (i.e. 30 μm for our MALDI stage) and also by the laser beam diameter (i.e. 100 μm for horizontal rastering). As shown in Figure III- 16.b-ii, the laser beam size is narrow enough to acquire MS image with pixels of 1x1 mm since it is 10-fold smaller than the pixels size; consequently, for a specific SRM transition, each pixel's intensity corresponds to the signal acquired in the pixel center (i.e. plain rectangle in Figure III- 16.b-ii). When acquiring MS images with higher resolution, the laser beam becomes the limiting factor, especially when sample stage incremental steps are smaller than the laser diameter (e.g. 50 μm steps, Figure III- 16.b-iii). In such cases the so-called strategy of "oversampling" has to acquire high resolution MS images.⁸⁵⁻⁸⁹ An example is shown in Figure III- 17 where the black and white SRM trace for COC acquired with pixels of 50x50 μm is overlaid to the optical image of the intact leaves (sample S3). At such resolution, small features of the leaves such as nerves can be revealed. It's noteworthy that the number of raster lines is also determining the total MS image acquisition time. Therefore, decreasing the Y-axis steps to improve MS image resolution will increase the overall analysis time. As a compromise between acquisition time and spatial resolution, pixels of 1x1 mm are sufficient to preliminary screen the targeted analytes on the intact leaves surface in *ca.* 140 minutes (i.e. image of 56.5 mm by 71 mm; 71 raster lines; acquisition time per line = 1 min. 53 sec. corresponding to the acquisition of the images shown in Figure S1 for whole leaves samples at stage 3). As a comparison, the 50- μm resolution image was acquired in 795 minutes only for the targeted region of 56.5 mm by 20 mm (Figure III- 17 for stage 3 samples).

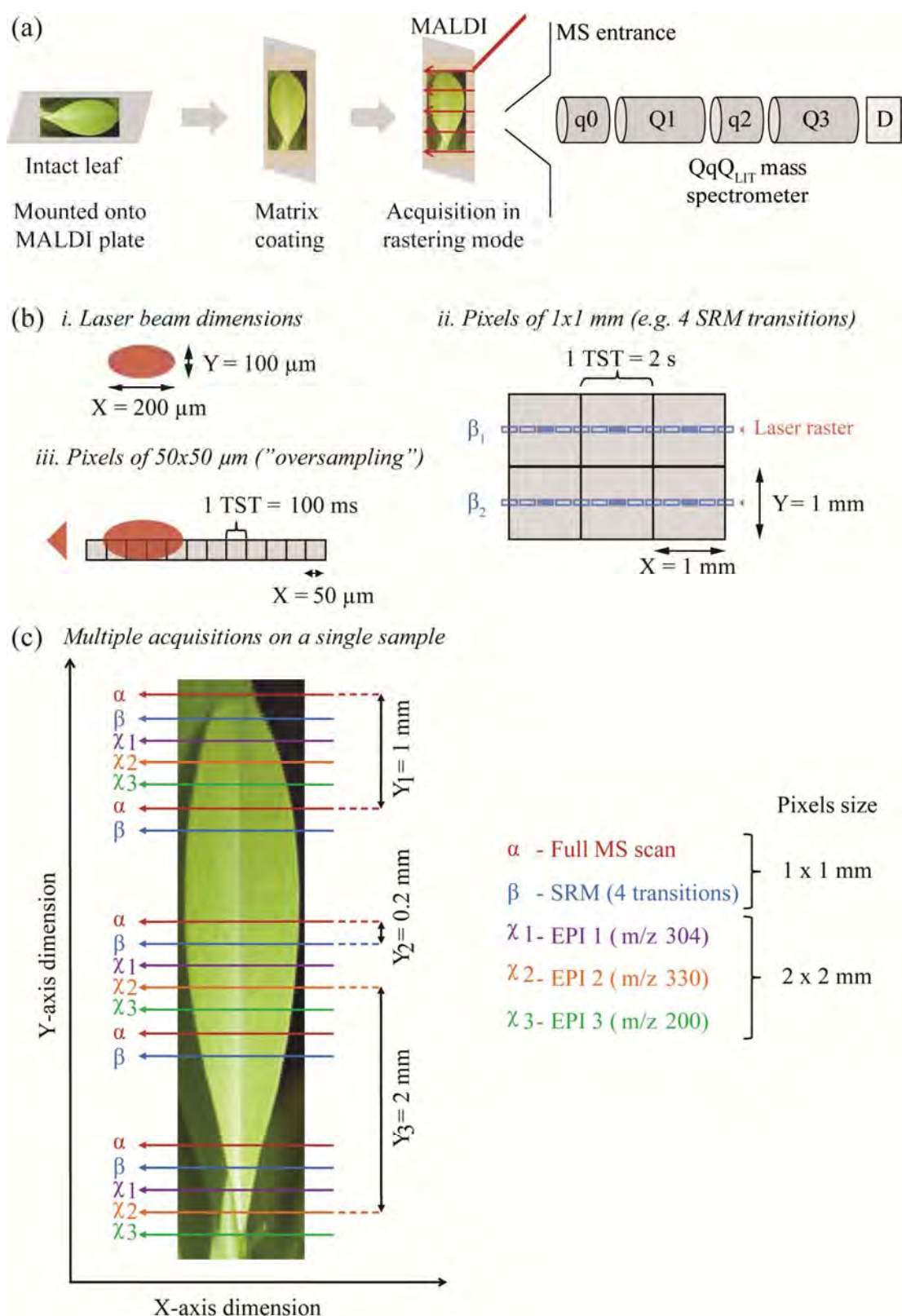


Figure III- 16. Direct analysis of intact *E. coca* leaves by MALDI-MS imaging. (a) MS imaging analytical workflow; (b) i) dimensions of the elliptical laser beam, schematic representation of: ii) pixels of $1 \times 1\ \text{mm}$ acquired by an SRM experiment with 4 transitions (β_1 and β_2 represent two successive laser raster lines, plain squares represent a specific SRM transition acquired every $1\ \text{mm}$), iii) pixels of $50 \times 50\ \mu\text{m}$ following the "oversampling" strategy; (c) experimental design for qualitative analysis with pixels of $1 \times 1\ \text{mm}$ and multiple acquisitions per sample. TST = total scan time, SRM = selected reaction monitoring, EPI = enhanced product ion.

Setup for multiple MS(/MS) acquisitions on a single sample. When generating images with pixels of 1x1 mm, only 10% of the sample is consumed during analysis since the laser beam dimension is of 100 μm (Figure S1b-ii). Therefore, and beside the gain of analysis speed, rastering the sample every 1 mm allows to perform multiple MS(/MS) experiments per sample. In this study, five different experiments were performed for a qualitative analysis of intact leaves: an unsupervised full MS scan, a targeted SRM experiment with four transitions related to the analytes of interest and three confirmatory MS/MS experiments (EPI) were acquired successively every 200 μm along the Y-axis (Figure III- 16.c). The full MS scan and SRM-based images were acquired with a distance of 1 mm between two raster lines giving images with pixels of 1x1 mm, however for the confirmatory analyses by EPI, a raster with Y-axis steps of 2 mm was acquired to decrease the analysis time (i.e. EPI MS images with pixels of 2x2 mm). After performing these preliminary screening experiments, it is possible to re-analyze the whole samples to generate higher resolution MALDI-SRM/MS-based images with pixels of 50x50 μm following the “oversampling strategy”, but the raster lines already analyzed during the first screening are almost giving no signal and generates horizontal dark lines in the MS images (Figure III- 17).

3.3.3.2. MALDI-MS imaging of cocaine, cinCOC and EME in intact *E. coca* leaves at different maturation stages

In order to observe accumulation patterns for methylecgonine, cinnamoylcocaine and cocaine, MALDI imaging techniques were employed. These three compounds were detected by selected reaction monitoring (SRM) acquisition mode and their identities further confirmed by enhanced product ion MS/MS experiments.

a) Detection and imaging of cocaine, cinCOC and EME in intact *E. coca* leaves

Upper side and lower side of leaves collected at different stages of maturity, i.e. stage 1 = rolled up leaves (S1), stage 2 = expanding leaves (S2), and stage 3 = mature leaves (S3), have been analyzed by MALDI mass spectrometry imaging.

Figure III- 17 shows the MALDI-SRM/MS images generated with pixels size of 50x50 μm and displays homogenous distribution for the three compounds in intact leaf samples of all leaf stages (S1, S2, S3). The cocaine signal was the highest of all three metabolite signals followed by cinnamoylcocaine and methylecgonine. Furthermore, there is a difference in signal intensity between the upper and lower surfaces of the leaf stages 2 and 3 (S2 and S3). The cocaine signal intensity is highest in the lower surface of leaf stage 2 and 3 (S2 and S3) compared to the upper leaf surface. In contrast, the signal intensity of cinnamoyl cocaine is higher in the upper surface of leaf stage 2 and 3 (S2 and S3). The methylecgonine signal intensity is overall very low, but is higher in the upper leaf surface of leaf stage 2 (S2) and the lower leaf surface of leaf stage 3 (S3) compared to the opposite leaf surface. An equal signal intensity distribution of all three metabolites can be observed in upper and lower leaf surfaces of leaf stage 1 (S1). The results are consistent with the SRM results obtained on the complete leaves surfaces where images were generated with pixels size of 1x1 mm (Figure S1).

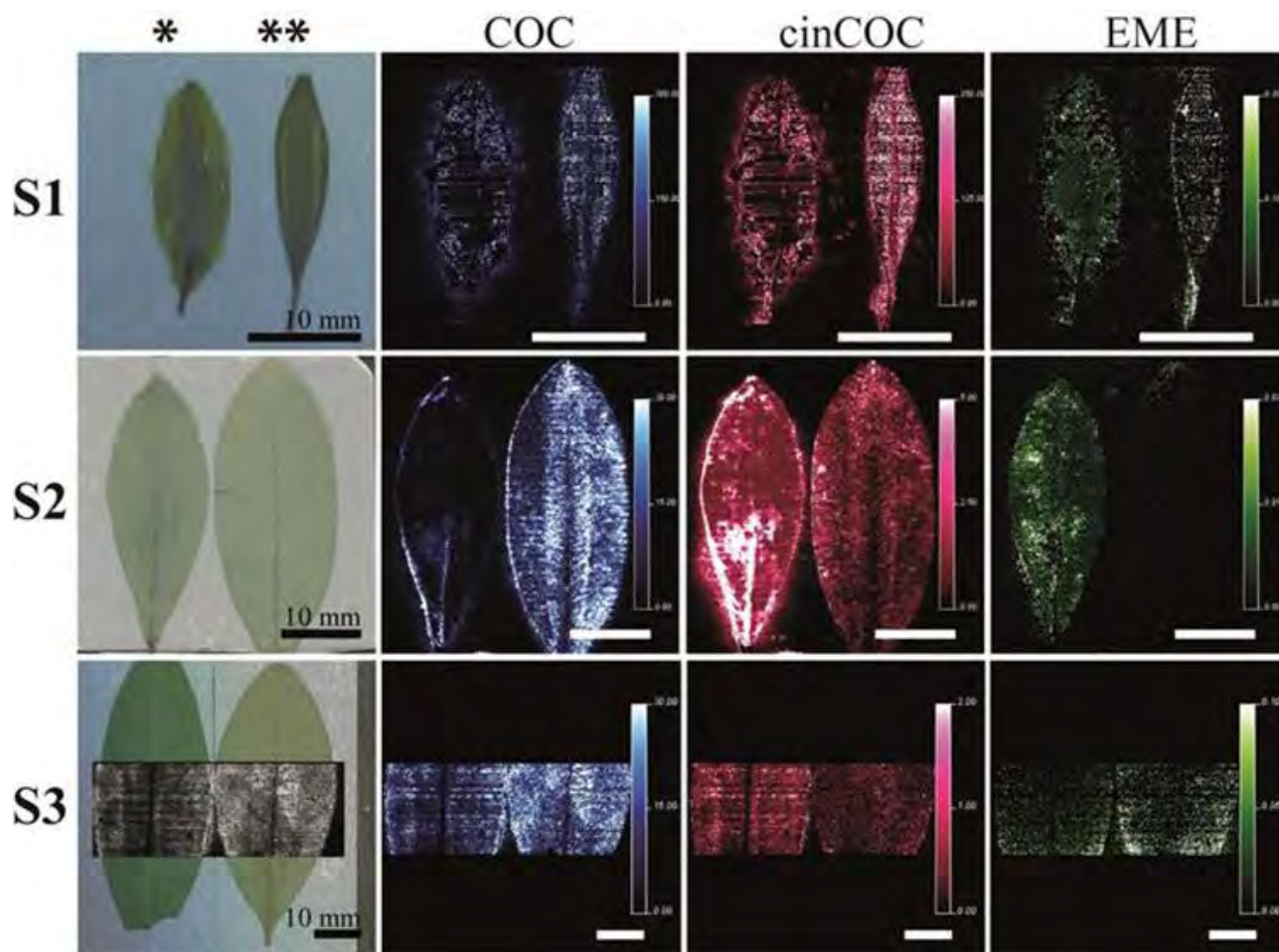


Figure III- 17. MALDI-SRM/MS images based on the respective SRM traces for targeted analytes: (a, blue trace) m/z 304 > m/z 182 for COC; (b, pink trace) m/z 330 > m/z 182 for cinCOC; (c, green trace) m/z 200 m/z 182 for EME in rolled up leaves (stage 1, S1), expanding leaves (stage 2, S2) and mature leaves (stage 3, S3). The images are displayed after normalization with the SRM trace of COC- d_3 . The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound (i.e. COC- d_3): white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. For S1: * = outside; ** = inside of rolled up leaf. For S2 and S3: * = upper side; ** = lower side of the leaves. MS image resolution: pixels of 50x50 μm .

b) MS/MS confirmation of the identity of the targeted compounds

Confirmation of the analytes identity is achieved by acquiring MS/MS images in the enhanced product ion (EPI) mode. The reference MS/MS spectrum obtained by the fragmentation of the cocaine precursor ion at m/z 304 displays characteristic cocaine fragment ions (m/z): 91, 105, 108, 119, 122, 150 and 182 (Figure S3.a). This confirms the presence of cocaine in rolled up (Figure S2.c), expanding (Figure S2.d) and mature leaves (Figure S2.e). The MS/MS spectra resulting from the fragmentation of the cinCOC precursor ion at m/z 330 displays the following fragments ions (m/z): 91, 103, 108, 119, 122, 131, 150, 182 and 200 and enables to clearly confirm the presence of cinCOC within the samples at the three stages of maturation (Figure S3). Finally, the confirmation of EME in stages 1 to 3 is performed by collecting fragment ions from the precursor ion at m/z 200 (i.e. m/z 103, 131, 150, 182 and 200) (Figure S4). EME spectra display more noisy spectra compared to those

obtained for COC and cinCOC due to the lowest response of the compound within the sample.

3.3.3.3. Supplementary analysis: MALDI-MS imaging of chloroform treated intact *E. coca* leaves at maturation stages S2 and S3

Direct analysis followed by mass spectrometry detection has been found particularly attractive in biomedical and biological applications since it tends to minimize the sample preparation and keeps intact the sample spatial integrity. Particularly, MALDI-MSI has been used to map plant metabolites within tissues^{81-82, 90} as well as to monitor specifically cocaine and its metabolites in single intact hair samples from humans for toxicological screening.⁴³ Mass spectrometry is particularly of interest as it allows for detecting individual tropane alkaloids based on their molecular weight as opposed to the less specific recognition abilities of the antibodies. To date, no other study using MALDI-MSI of plant leaf tissues has reported differences between the upper and lower leaf surfaces.⁹¹⁻⁹⁴ The upper surface of coca leaves has a thick waxy cuticle containing hentriacontane as one of the major lipid components.⁹⁵ Wax components in the cuticle of *A. thaliana* are known to decrease the abundance of ions corresponding to flavonoids present within the leaf.⁹² In order to remove the wax for further study, the Arabidopsis leaves were dipped in chloroform. In our case, chloroform treatment of leaf stage 2 and 3 leaves (i.e. immersion for 5 second before being frozen in liquid nitrogen and stored at -80°C prior to the analysis) did not change distribution pattern from upper and lower epidermis, but it should be noted that the amount of cuticle wax removed was not determined (Figure S5.b,c).

Observed differences in MS signal ratio intensities between both sides of the intact or treated leaves do not take into account possible matrix effects (i.e. ionization suppression or analyte recovery) due to the difference in wax layer thickness or other endogenous compounds present. Despite the additional experiments to remove surface wax by dipping the leaves into chloroform, tropane alkaloid could still be identified on both sides of the leaves but no relative quantification between lower or upper leaf surfaces could be achieved. Nevertheless the distribution pattern of tropane alkaloids remains evenly distributed across the leaf in *E. coca* which was also confirmed by recording the MS/MS spectrum and by comparison with authentic standards. In contrast, the tropane alkaloids scopolamine and atropine are mainly found in the vascular tissues of *D. stramonium* leaves (Solanaceae) when analyzed by indirect desorption electrospray (DESI).

3.3.4. Conclusion

The tropane alkaloids cocaine, cinnamoylcocaine and ecgonine methyl ester have been analyzed by MALDI mass spectrometry imaging in *Erythroxylum species*. Experiments have been performed both in full MS scan and selected reaction monitoring acquisitions and the compounds identity have been confirmed by enhanced product ion MS/MS experiments. The results display homogenous distribution of COC, cinCOC and EME in intact leaf samples in early stage of development of the plant (S1) as well as in expanding leaves (S2) and mature stage (S3), with predominant signals coming from the lower epidermis compared to the upper epidermis.

At this point, observed differences in signal ratio intensities between both sides of the leaves do not take into account possible ionization effects (i.e. matrix suppression) due to the different wax layer thickness or other endogenous compounds distribution. The amount of wax removed after treatment with chloroform cannot be estimated so far and we cannot exclude that a different ionization suppression effect may still occur from one face to another. The current method does not allow determining accurately extraction efficiency of the analyte from deeper layers of the epidermis of the leaf samples.

3.3.5. Perspectives

The only way to determine accurately the distribution of tropane alkaloids is to analyze cross-sections of leaves. For that purpose, time-of-flight secondary ion mass spectrometry (ToF-SIMS) has been used.

The sample preparation consisted in embedding intact fresh leaf from the stage 3 (mature leaf) have in 2% carboxymethylcellulose, and flash-frozen in liquid nitrogen, and further cryosectioned the leaves at 12 μm -thickness, and directly mounted in ITO glass slide, and coated with a thin layer of gold.

Preliminary ToF-SIMS results display the distribution of tropane alkaloids mainly in the upper epidermis and in the mesophyll in the central nerve of the leaf, which correlates immunolabeling results (Figure S6.a). The high-resolution ToF-SIMS image (i.e. pixel of 1 μm x 1 μm) is based on the signal given by the ion at m/z 182 (Figure 6.b). Due to extensive fragmentation caused by the primary ion beam in SIMS, this ion at m/z 182 is predominant in the mass spectrum, and only cocaine and ecgonine methyl ester protonated ions have been detected, but not cinnamoylcocaine, in opposition to previous MALDI-SRM/MSI results.

In addition, it is noteworthy that the optimization of the sample preparation has to be carried out. This step is really critical, because leaves are rather fragile samples, easily breakable. During the sectioning step, only the central nerve could be cut intact, while the wings of the leaves were damaged. The objective of further analysis will be to carried out ToF-SIMS imaging on the whole cross-section, from the three stages of maturity and to correlate immunolabeling results.

3.3.6. Supplemental material

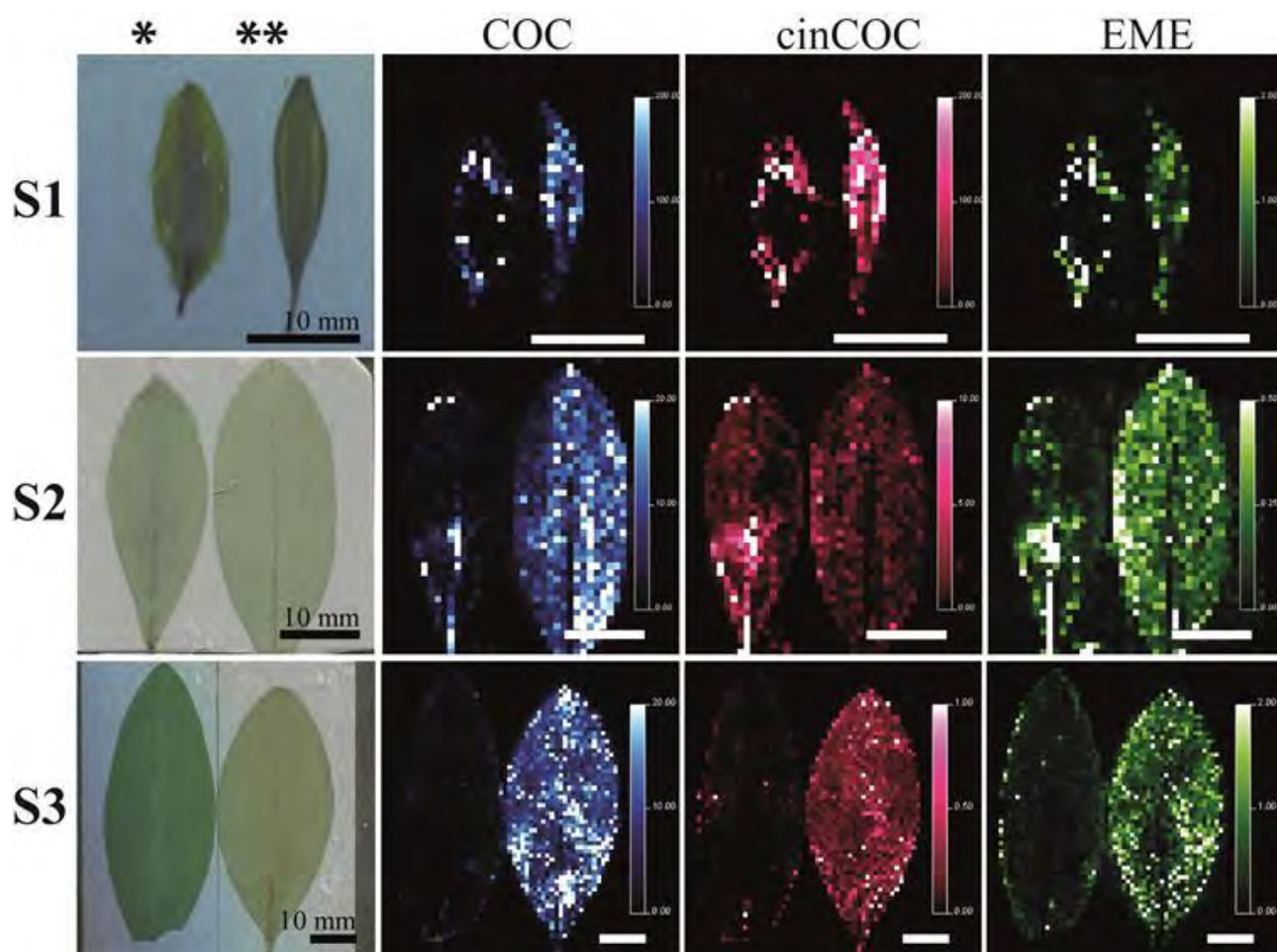


Figure S1. MALDI-SRM/MS images with pixels of 1x1 mm. The images are obtained after monitoring the following SRM traces: (a, blue trace) m/z 304 > m/z 182 for COC; (b, pink trace) m/z 330 > m/z 182 for cinCOC; (c, green trace) m/z 200 m/z 182 for EME in rolled up leaves (stage 1, S1), expanding leaves (stage 2, S2) and mature leaves (stage 3, S3). The images are displayed after normalization with the SRM trace of COC- d_3 . The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound (i.e. COC- d_3): white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. For S1: * = outside; ** = inside of rolled up leaf. For S2 and S3: * = upper side; ** = lower side of the leaves.

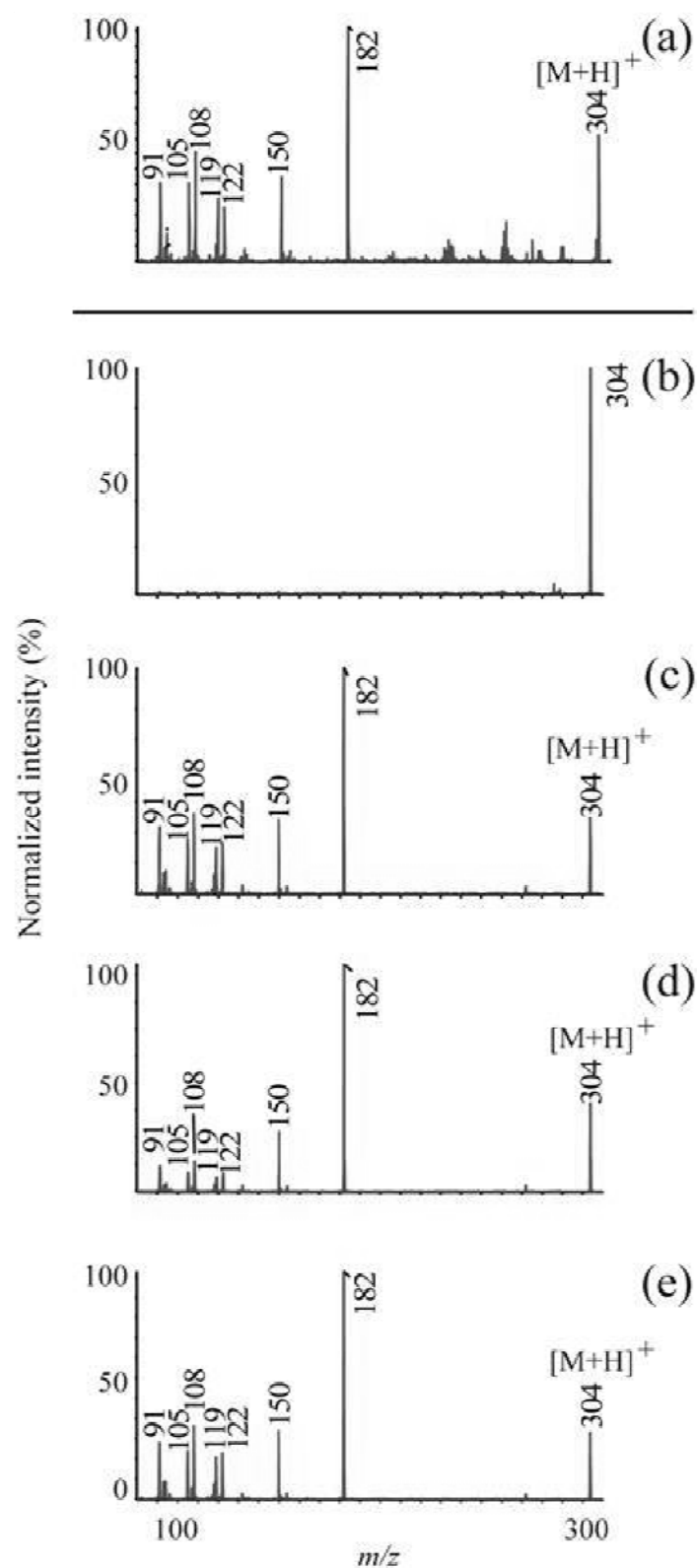


Figure S2. MALDI-MS/MS spectra for cocaine acquired in EPI mode of the precursor ion at m/z 304. (a) reference spectrum from a MALDI spot containing 100 pg of standard. The following spectra are generated by summing 6 vertical adjacent pixels over selected regions: (b) blank spectrum acquired from a region out of the leaf and corresponding to background generated from CHCA; (c) in stage 1 (rolled up leaves) sum of 4x6 pixels; (d) in stage 2 (expanding leaves) sum of 4x6 pixels; and (e) in stage 3 (mature leaves) sum of 4x10 pixels. Collision energy of 35 eV, trap fill time of 300 ms.

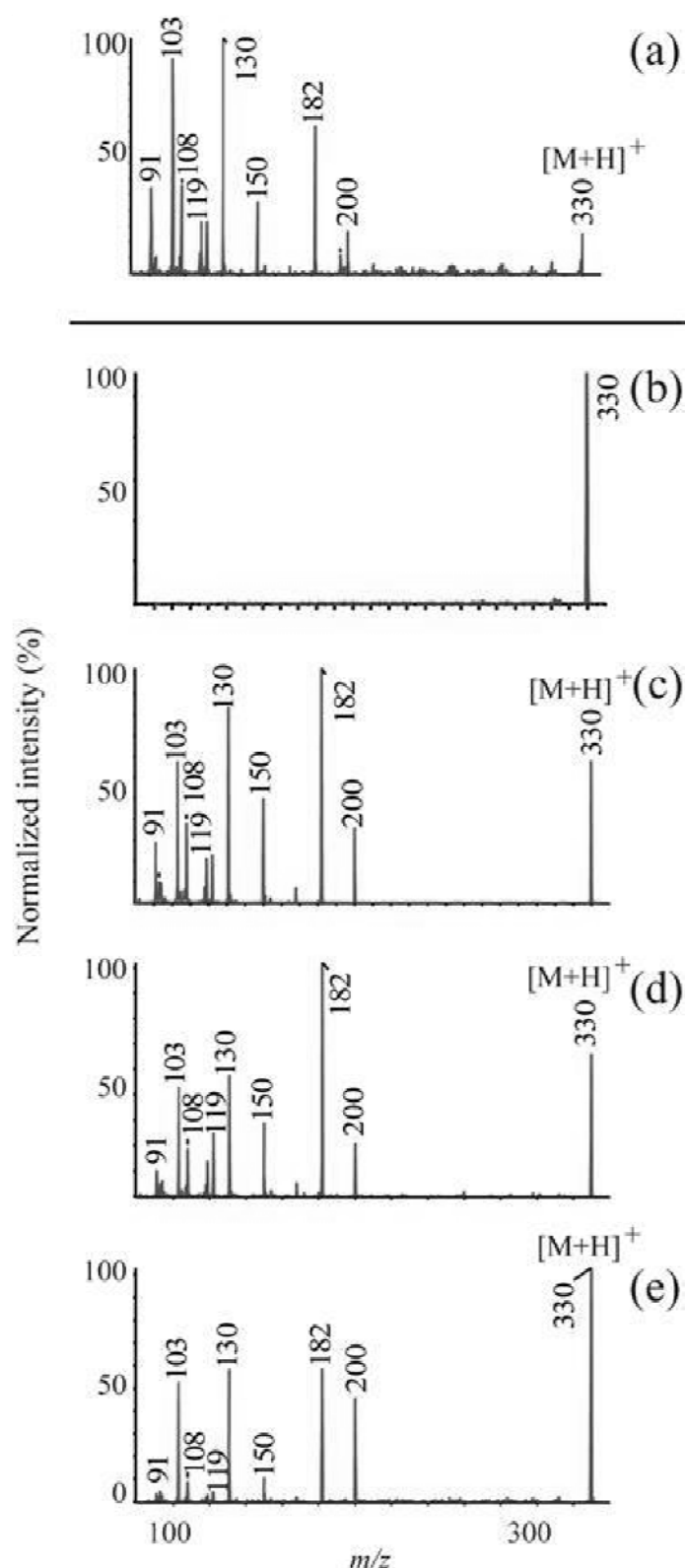


Figure S3. MALDI-MS/MS spectra for cinCOC acquired in EPI mode of the precursor ion at m/z 330. (a) reference spectrum from a MALDI spot containing 100 pg of standard. The following spectra are generated by summing 6 vertical adjacent pixels over selected regions: (b) blank spectrum acquired from a region out of the leaf and corresponding to background generated from CHCA; (c) in stage 1 (rolled up leaves) sum of 1x6 pixels; (d) in stage 2 (expanding leaves) sum of 6x8 pixels; and (e) in stage 3 (mature leaves) sum of 4x10 pixels. Collision energy of 40 eV, trap fill time of 300 ms.

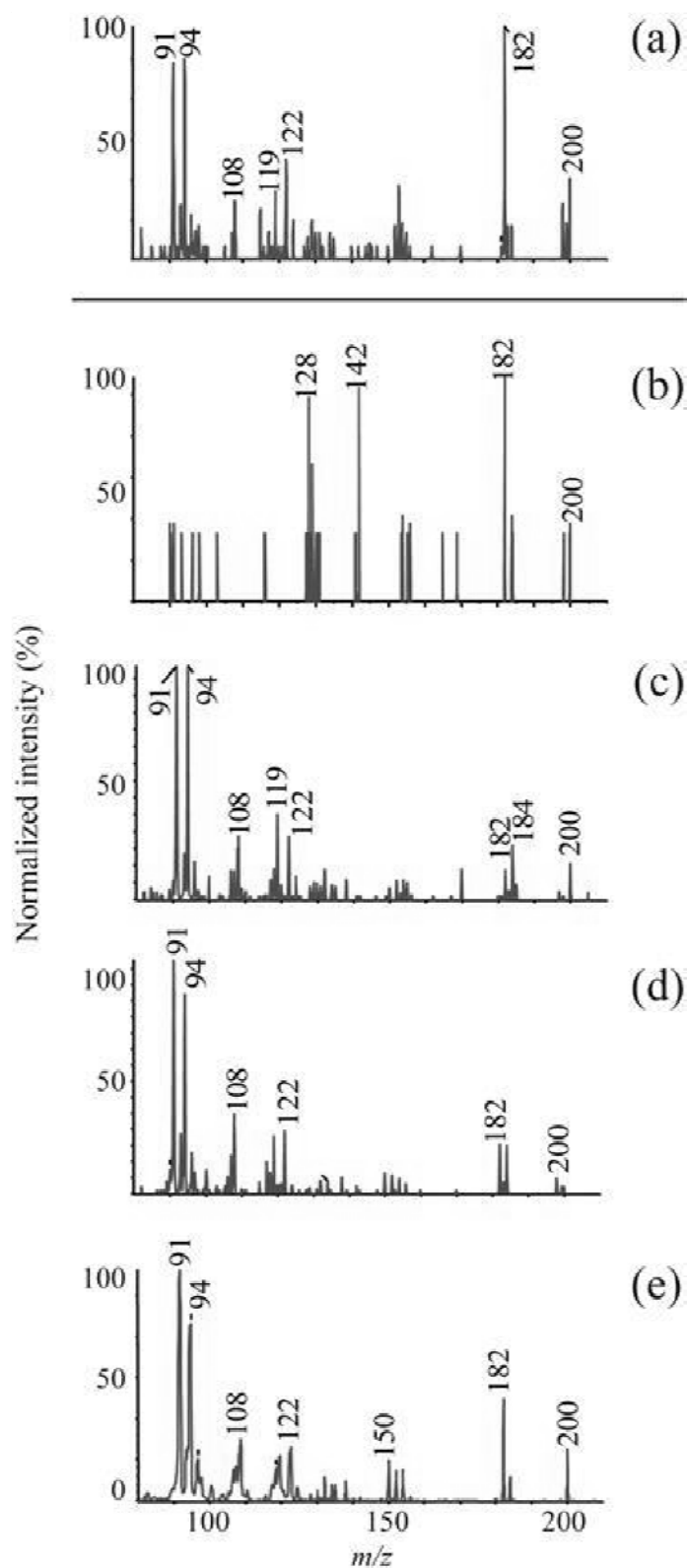


Figure S4. MALDI-MS/MS spectra for EME acquired in EPI mode of the precursor ion at m/z 200. (a) reference spectrum from a MALDI spot containing 100 pg of standard. The following spectra are generated by summing 6 vertical adjacent pixels over selected regions: (b) blank spectrum acquired from a region out of the leaf and corresponding to background generated from CHCA; (c) in stage 1 (rolled up leaves) sum of 1x6 pixels; (d) in stage 2 (expanding leaves) sum of 2x6 pixels; and (e) in stage 3 (mature leaves) sum of 7x10 pixels. Collision energy of 40 eV, trap fill time of 350 ms.

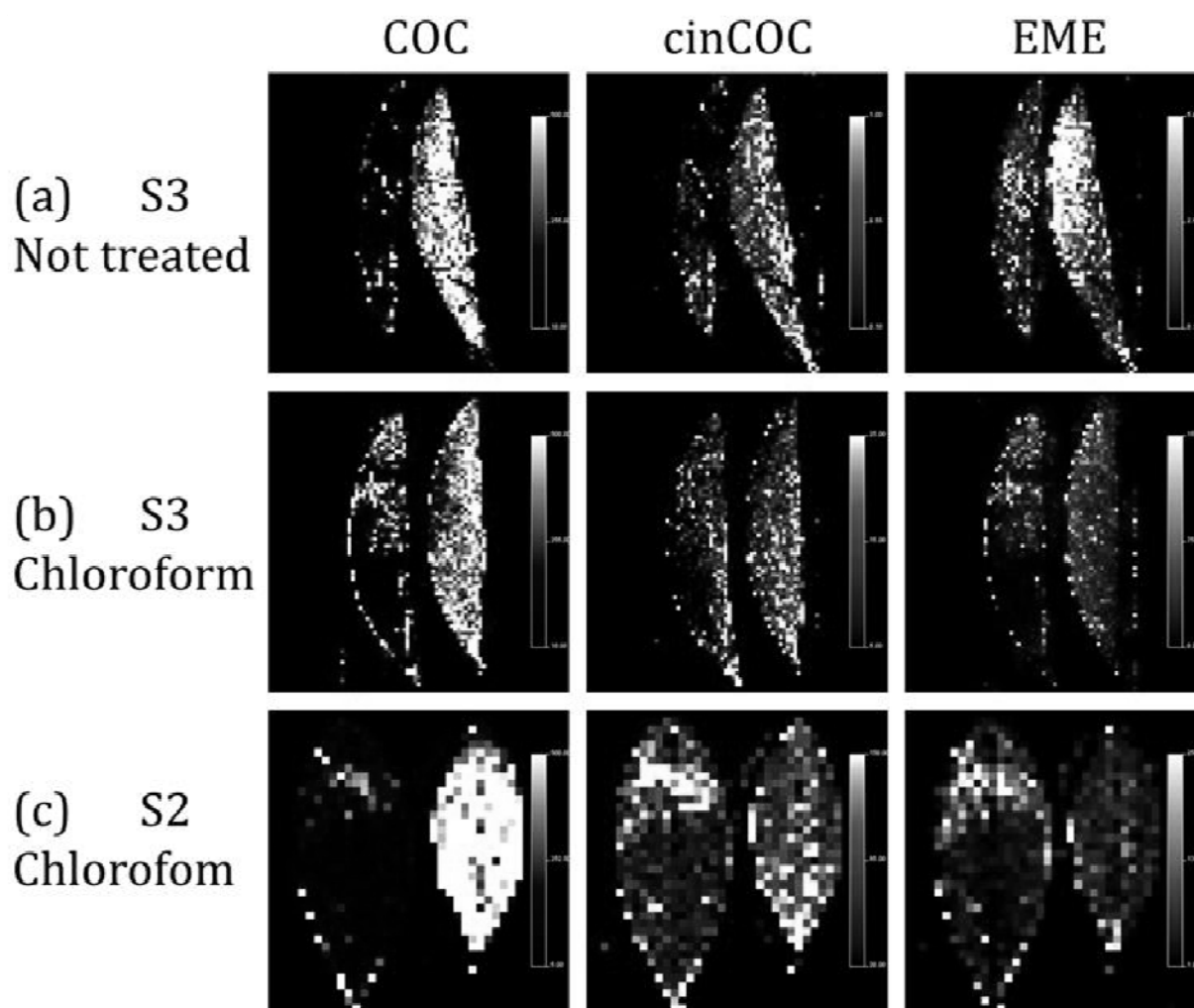


Figure S5. MALDI-SRM/MS of *E.coca* leaves (a) before and (b,c) after treatment with chloroform to remove the waxy cuticle. The images are based on the respective SRM traces for targeted analytes: m/z 304 > m/z 182 for COC (blue trace); m/z 330 > m/z 182 for cinCOC (pink trace); m/z 200 > m/z 182 for EME (green trace) in (a,b) mature leaf (stage 3, S3) and (c) expanding leaves. (a,b) a leaf specimen was cut into 2 pieces along the central nerve, so that both upper (left side of each image) and lower (right side) epidermis could be analyzed. The images are displayed after normalization with the SRM trace of COC- d_3 . The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound (i.e. COC- d_3): white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. For S1: * = outside; ** = inside of rolled up leaf. For S2 and S3: * = upper side; ** = lower side of the leaves. MS image resolution: pixels of 1x1 mm.

Comment: automation of the spraying of the matrix

A method has been developed to perform the matrix deposition in an automated fashion to achieve controllable, homogeneous and reproducible matrix deposition from one analysis to another. The automation of the spray has been performed to better control the homogeneity of the matrix coating (Figure S5.a). For the spraying of the spray automated, the airbrush was fixed on two axis-stages motors (models A-LSQ300B [horizontal axis] and A-LSQ150B [vertical axis], Zaber Technologies Inc., Vancouver, BC, Canada), to allow for controlling the sprayer motion and speed along a fixed axis. The sprayer was placed at a distance of 25 cm from the target plate and moved linearly at a constant speed of 15 mm/s. 30 layers of CHCA matrix solution containing the internal standard (solution of 200 ng/mL) were applied. To validate the results, an intact *E. coca* leaf was cut into 2 pieces along the central nerve, so that both upper and lower epidermis could be analyzed. This is possible because previous results enabled us to determine the homogenous distribution of cocaine all over the leaves. Figure S5.a displays the results for untreated leaf from the stage of maturation S3 and displays an overall signal which is higher from the lower epidermis compared to the upper epidermis for the 3 targeted compounds, which confirms previous results observed after the manual spraying of the matrix.

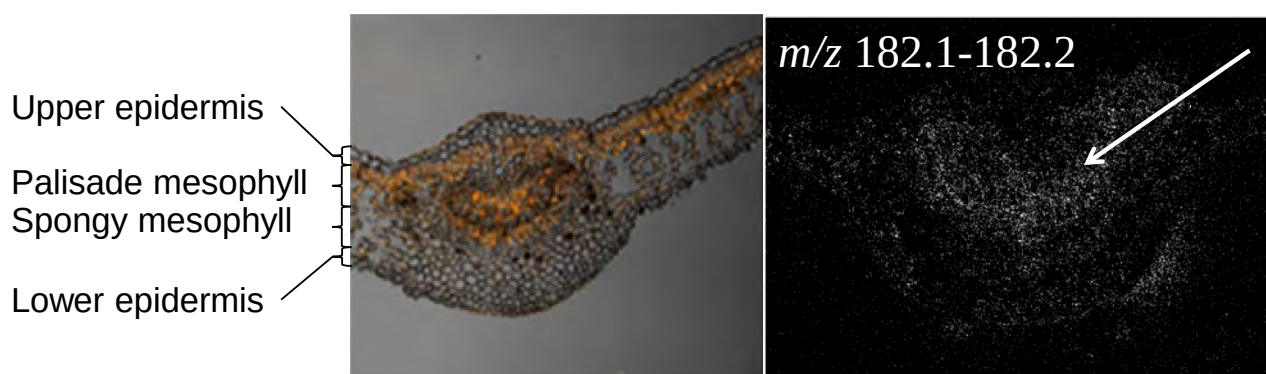


Figure S6. Immunolabeling of cocaine in cross-sections of *E. coca* leaf stage 3 (left) and high resolution ToF-SIMS imaging. The samples were labeled with anti-cocaine antibodies. ToF-SIMS imaging displays the distribution of the ion at m/z 182, which is a fragment ion characteristic for tropane alkaloids such as cocaine and ecgonine methyl ester, mainly distributed in the upper epidermis and in the mesophyll. Immunolabeling and ToF-SIMS imaging results correlate well. Immunolabeling from ref.⁸⁰

CHAPTER 3.

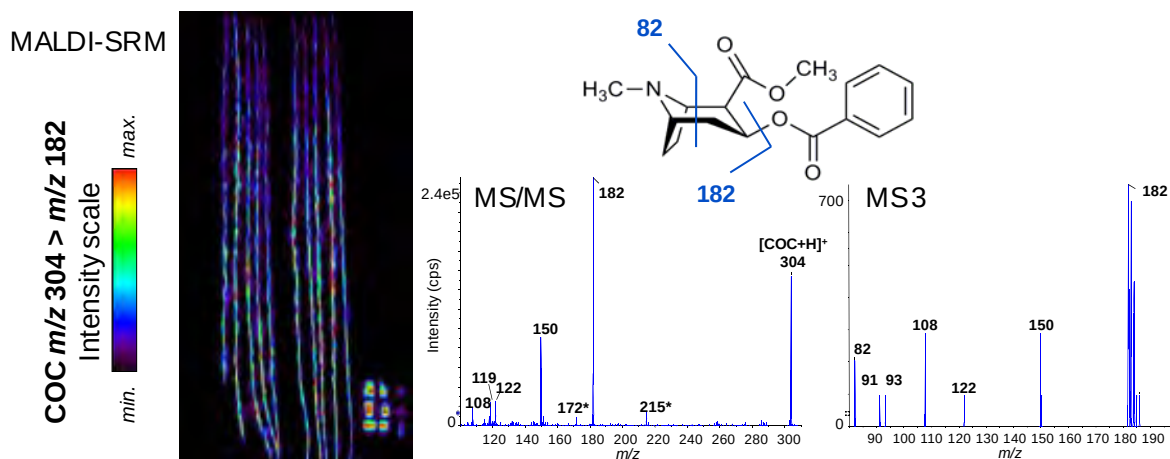
Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 4 – Multimodal analysis of cocaine in single hair sample by combining fast acquisition MALDI-SRM/MS and high resolution ToF-SIMS imaging

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 4a – Multimodal analysis of cocaine in single hair sample: Fast acquisition by MALDI-SRM/MS



Adapted from:

Porta T., Grivet C., Kraemer T., Varesio E. and Hopfgartner G., Single hair cocaine consumption monitoring by mass spectrometric imaging. *Anal. Chem.* **2011**, 83 (11), 4266-4272

Abstract

Matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) was used to image the distribution of cocaine and its metabolites in intact single hair samples down to a concentration of 5 ng/mg. Acquisitions were performed in rastering mode, at a speed of 1mm/sec and in the selected reaction monitoring mode (SRM) on a MALDI triple quadrupole linear ion trap fitted with a high repetition rate laser (1 kHz). Compared to traditional methods based on LC-MS/MS or GC-MS(/MS) which require to segment the hair to obtain spatial resolution, MALDI-MSI, with a straightforward sample preparation beforehand, allowed obtaining a spatial resolution of 1 mm and thus the chronological information about cocaine consumption contained in a single intact hair over several months could be monitored. The analysis time of an intact single hair sample of 6 cm is approximately of 6 minutes. Cocaine and its metabolites benzoylecgonine, ethylcocaine and norcocaine were investigated in nine sets of hair samples for forensic purpose. The analyses were accomplished by spraying α -cyano-4-hydroxycinnamic acid (CHCA) or 4-chloro- α -cyano-cinnamic acid (Cl-CCA) as MALDI matrices. We also propose a rapid strategy for sensitive confirmatory analyses with both MS/MS (enhanced product ion) and MS³ experiments performed directly on intact hair samples. Since only part of the hair strand is analyzed, additional analyses are possible at any time on the remaining hair from the strand.

Key words

Hair analysis, mass spectrometric imaging, forensics, drugs of abuse, cocaine

3.4a.1. Introduction

In toxicological or forensic investigations, hair testing has been demonstrated as a powerful tool for the confirmation of drugs of abuse (DOA) consumption.⁹⁶⁻⁹⁹ Indeed, hair samples provide several advantages such as i) high stability of the analyte from weeks to years, ii) non-invasive sample collection, and iii) a wider time detection window which enables the investigation of the long DOA intake history compared to blood, urine or oral fluid which only provide general information on a recent drug use. Drugs of abuse hair testing is generally performed on few cm-length hair segments and the relative drug quantification over several months (*i.e.* several segments) helps to characterize an addicted behavior, a decreased consumption,¹⁰⁰ but also a single dose.¹⁰¹ To date, various strategies based on gas or liquid chromatography coupled with mass spectrometric detection have been developed to analyze DOA in hair.^{97, 102-104} Nevertheless, these approaches usually require extensive sample preparation protocols,^{97, 102} including several time-consuming steps, such as pulverization, overnight hair digestion, derivatization, liquid-liquid extraction or solid-phase extraction and multiple injections for a single sample.

Typical cocaine concentrations found in hair samples range from 0.5 to 216 ng/mg.¹⁰⁵ Vogliardi and co-workers have described a fast screening method for the detection of cocaine in hair by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).¹⁰⁶⁻¹⁰⁷ After extraction of hair samples by incubation in methanol/trifluoroacetic acid (15 min at 45°C), a sensitive analysis was achieved by using a MALDI spot preparation based on a multi-layer approach. The spot preparation consisted of a graphite layer, the sample (0.2 mg of powdered hair samples) and electrosprayed α -cyano-4-hydroxycinnamic acid (CHCA) matrix on the top. With this sample preparation, a limit of detection of 0.1 ng/mg could be reached for cocaine. However in the same study, the direct MALDI-TOF analysis on an intact single hair (4-cm segment) containing 100 ng/mg of cocaine was also investigated, but no valid results were obtained due to the lack of sensitivity and many matrix-derived interfering ions in the low m/z range. Furthermore, Reich *et al.* have used an intermediate pressure MALDI source mounted on a linear ion trap to quantify cocaine in post-mortem human brain tissue (0.35 ng/mg of brain tissue) using MSⁿ experiments.¹⁰⁸

Recently, MALDI with a high repetition laser combined to a triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer has been described as a powerful analytical platform for the quantitative and qualitative analysis of low molecular weight compounds (LMWC) in biological fluids such as plasma or in tissues.^{1, 39, 49-50, 109} The MALDI-SRM/MS was found to be particularly sensitive for targeted analysis without any chromatographic separation prior detection. Tandem mass spectrometry is almost mandatory for sensitive MALDI analysis of LMWC because it eliminates precursor ion matrix-derived interferences. In case of cross-talk, alternative matrices to CHCA have been recently described to tune background interfering matrix ions.³⁰

Moreover, the combination of MALDI with a high repetition rate laser allows to collect spatial MS images (MALDI-MSI) within minutes to hours, depending on the surface to

sample. MALDI-MSI was initially applied to peptides and proteins analysis¹¹⁰⁻¹¹¹ and more recently to pharmaceuticals and metabolites profiling in rodents.^{1, 35}

We present herein a mass spectrometric method which focuses on the rapid MS imaging and the relative quantification of cocaine and its metabolites in a single hair based on MALDI triple quadrupole linear ion trap platform (MALDI-QqQ_{LIT}). The QqQ_{LIT} instrument offers unique capabilities to perform simultaneously target quantification in SRM mode and sensitive MS/MS and MS³ acquisition for confirmatory analyses.^{46, 112-114}

3.4a.2. Experimental section

3.4a.2.1. Chemicals and reagents

Cocaine (COC), cocaine-d3 (COC-d3), benzoylecgonine (BZE), benzoylecgonine-d3 (BZE-d3), ethyl-cocaine (ECOC) and ethyl-cocaine-d3 (ECOC-d3) were provided by Lipomed (Arlesheim, Switzerland). Norcocaine (NCOC) and norcocaine-d3 (NCOC-d3) were provided by Cerilliant (Molsheim, France). Methanol (MeOH, HPLC grade) was provided by Sigma-Aldrich (Buchs, Switzerland). α -cyano-4-hydroxycinnamic acid (CHCA) was provided by Fluka (Buchs, Switzerland). Formic acid (HCOOH) was purchased from Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

3.4a.2.2. Standards and matrix solutions

Stock solutions of COC, BZE, ECOC and NCOC were prepared at a concentration of 1 mg/mL in MeOH and stored at 4°C prior the analysis. Then, standard solutions of COC (2-150 pg/ μ L), BZE (1-75 pg/ μ L), ECOC (1-50 pg/ μ L) and NCOC (1-50 pg/ μ L) were individually prepared in MeOH:H₂O (60:40, v/v) and 1 μ L was spotted onto the plate for MS analysis. Solutions of deuterated standards (*i.e.* 10 ng/mL of COC-d3, BZE-d3, ECOC-d3 and NCOC-d3) were added to matrix solution and sprayed simultaneously with the matrix.

CHCA and Cl-CCA were dissolved at a concentration of 10 mg/mL in appropriate solvent mixture (MeOH:H₂O:HCOOH, 60:40:0.1, v/v/v). Due to its low solubility, NpCCA was dissolved at a concentration of 4 mg/mL in MeOH:H₂O:HCOOH, (70:30:0.1, v/v/v). Matrix solutions were kept in amber glass bottles to prevent their degradation by light and stored at 4°C between uses.

3.4a.2.3. Sample preparation of hair samples for routine LC-SRM/MS

Head hair samples from 9 different individuals were collected at the Department of Forensic Pharmacology and Toxicology (Institute of Legal Medicine) of the University of Zurich. For LC-SRM/MS analysis, the routine protocol for drugs of abuse analysis in the Institute of Legal Medicine in Zurich comprises a three steps washing procedure with water (2 minutes shaking, 15 ml), acetone (2 min, 10 ml) and finally hexane (2 min, 10 ml). The hair samples were dried at room temperature. Then, the hair samples were cut into small snippets and extracted in two steps, first with MeOH (5 ml, 16 hours, ultrasonication) and a second step with 3 ml MeOH acidified with 50 μ L hydrochloric acid 33 % (3 hours, ultrasonication). The

extracts were dried and the residue reconstituted with 50 μ L MeOH and 500 μ L 0.2 mM ammonium formate (analytical grade) in water. COC-d3, BZE-d3 and ECOC-d3 were used as internal standards.

3.4a.2.4. Sample preparation of intact hair samples for MALDI-MS

The washing procedure for a single hair sample consists in washing in water, acetone and finally hexane (each step: 30 seconds shaking, 20 mL). After drying at room temperature, the intact washed hair samples were fixed onto the stainless steel MALDI plate using a double-sided adhesive foil. Approximately 12 mL of CHCA or Cl-CCA 10 mg/ml-solutions (or 15 mL for NpCCA 4 mg/ml-solution) were applied to the MALDI plate (OPTI TOF 384 well Insert - 123 x 81 mm, AB Sciex, Concord, Canada) by manual airspray using a TLC sprayer (Sigma-Aldrich, Buchs, Switzerland) at a distance of 30 cm. The nebulizer gas pressure was kept constant at 0.2 bar.

3.4a.2.5. SRM transitions optimization by infusion

MS/MS spectra acquisition was performed in positive polarity on a triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer (4000 QTRAP, AB Sciex) fitted with an electrospray ionization (ESI) source. 50 pg/ μ L sample solutions in 0.1% formic acid in MeOH:H₂O (50:50, v/v) were infused at a flow rate of 15 μ L/min. Conditions were: ion spray voltage = 4.8 kV; curtain gas = 10 psi; entrance potential EP = 10 V; collision cell exit potential = 15 V. Nitrogen was used as collision gas (vacuum gauge in q_2 = $3.6 \cdot 10^{-5}$ Torr). The SRM dwell time was 50 ms per transition. The optimized SRM transitions are detailed in Table III- 7. The analyzer resolution was set to unit for both Q₁ and Q₃ analyzers.

Table III- 7. SRM transitions for cocaine, its metabolites and the deuterated standards. PI = precursor ion; FI = fragment ion; DP = declustering potential; CE = collision energy. In bold, the most intense transition which is monitored. * Common FI for BZE/NCOC and BZE-d3/NCOC-d3.

Analyte	PI (m/z)	FI (m/z)	DP (V)	CE (eV)
COC	304.2	182.1	65	27
COC-d3	307.2	185.1	65	27
ECOC	318.2	196.1	65	27
ECOC-d3	321.2	199.1	65	28
BZE	290.2	168.1*	70	26
	290.2	105.0	70	45
BZE-d3	293.1	171.1*	70	28
	293.1	153.0	70	35
NCOC	290.2	168.1*	55	23
	290.2	136.0	55	33
NCOC-d3	293.1	171.1*	55	23
	290.2	136.0	55	33

3.4a.2.6. LC-SRM/MS routine analysis

Hair samples used in this study were previously analyzed at the Institute of Legal Medicine of Zurich by LC-SRM/MS. LC-SRM/MS analyses were performed on a Q TRAP 3200 instrument fitted with a Turbo V ion source operated in the ESI mode (AB Sciex) and coupled with a Prominence HPLC system consisting of a CBM 20A controller, two LC 20AD pumps including a degasser, a SIL 20AC autosampler and a CTO 20AC column oven (Shimadzu, Duisburg, Germany). MS operating conditions were: gas 1 (nitrogen) = 50 psi; gas 2 (nitrogen) = 60 psi; curtain gas (nitrogen) = 20 psi; ion spray voltage = 3'500 V; ion source temperature = 450 °C. The analysis was performed in SRM mode with two transitions per analyte and one transition for each deuterated internal standard, respectively. Gradient elution was performed on a Synergi Polar-RP narrow-bore column (150 x 2.0 mm, 4µm / 80A) equipped with a POLAR-RP 4 x 2.0 guard cartridge (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of 1 mM ammonium formate buffer adjusted to pH 3,5 with formic acid (eluent A) and acetonitrile containing 1 mM ammonium formate and 1 mM formic acid (eluent B). The quantitative results from LC-SRM/MS routine analysis in the 9 different hair samples are given Table III- 8.

Table III- 8. Quantitative results from routine LC-SRM/MS analyses for different hair samples. S = segment. n.a. = data not available. The quantities are given in ng /mg. Samples H1 and H2 (marked in bold) were used as reference samples as they were determined as containing no drugs.

# of sample	H1	H2	H3	H4	H5		H6	
Segment	S1	S1	S1	S1	S1	S2	S1	S2
Length (cm)	4	3.5	3	3	1	4	3	3
COC (ng/mg)	0	0	0.26	0.33	4.90	8.50	6.70	40
BZE (ng/mg)	0	0	0.02	0.11	0.50	0.75	2.70	12
ECOC (ng/mg)	0	0	0	0.02	0.25	0.50	0.38	1.30
NCOC (ng/mg)	0	0	0	0.02	0.25	0.20	0.15	0.80

# of sample	H6		H7	H8		H9
Segment	S1	S2	S1	S1	S2	S1
Length (cm)	3	3	6	2.5	2.5	6
COC (ng/mg)	6.70	40	130	99	170	230
BZE (ng/mg)	2.70	12	12	13	20	44
ECOC (ng/mg)	0.38	1.30	12	0	0	3
NCOC (ng/mg)	0.15	0.80	n.a.	0.73	1	n.a.

3.4a.2.7. MALDI-MS imaging

MS and MS/MS images were acquired in positive ionization mode on a MALDI-4000 QTRAP instrument (AB Sciex) fitted with a frequency-tripled Nd:YAG laser 355 nm (elliptic beam shape of 200 by 100 µm). General operating conditions were: data acquisition mode = rastering, repetition rate laser = 1000 Hz; laser energy = 60 µJ; MALDI source and q₀ region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q₂ = 2.4 10⁻⁵ Torr (nitrogen used as collision gas); declustering potential = 80 V; entrance potential = 10 V;

quadrupole resolution was set to unit. Exhaustive operating conditions for each MS mode are described in Table III- 9.

Table III- 9. Operating MS conditions. SRM = selected reaction monitoring mode. EPI = enhanced product ion. X-step = distance between two adjacent rastered lines.

Mode	Full Scan (Q1 mode)			SRM	MS/MS (EPI mode)			MS ³
	<i>m/z</i>	100	-		<i>m/z</i>	100	-	<i>m/z</i> 100 -
Scan range	1000			Fixed	310			200
Scan rate (u/s)	500			-	1000			4000
LIT fill time (ms)	-			-	300			500
q ₀ trapping	-			-	-			on
Plate speed (rastering mode) (mm/s)	0.125			1	0.063			0.063
X-step (mm)	1			1	-			-
# transitions monitored	-			10	-			-
Dwell time (ms)	-			5	-			-
Period cycle time (s)	2			0.1	1.95			0.82
Q3 entry barrier (V)	8			-	8			8
Excitation energy (AF2)	-			-	-			20
Excitation time (ms)	-			-	-			100
Analysis time for a single hair	<i>c.a.</i> 47 min ¹			Within min ¹	6	<i>c.a.</i> 1 min ²		<i>c.a.</i> 2 min ²

¹: considering a region of 70 x 5 mm containing a single hair of 6 cm.

²: considering a 2 mm-hair segment.

3.4a.2.8. Software for data acquisition and processing

The MALDI source and its laser were controlled using a custom M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control, data collection. PeakView software (v.1.0.0.3, AB Sciex) was used for raw data processing (.wiff files). A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files (.wiff format) into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex) used for MS images generation and processing.

3.4a.3. Results and discussion

3.4a.3.1. MALDI-MS hair sample analytical workflow

While several steps are needed prior to LC-MS or GC-MS analyses (*i.e.* pulverization, overnight hair digestion, dedicated extractions, derivatization, etc.^{97, 102}), MALDI-MS hair sample analysis follows a straightforward sample preparation and analytical workflow (Figure III- 18). The first step consists in the wash of intact hair samples to avoid external contamination which may come from sebum, sweat, substance powder, etc. and may lead to false positive results. Then, each intact and washed hair is placed onto an adhesive foil preliminary fixed on the MALDI plate. The MALDI matrix, *i.e.* CHCA or Cl-CCA dissolved at 10 mg/mL in an acidified methanolic solution, is applied by manual airspray with a TLC sprayer. The coating step lasts from 30 to 40 minutes depending on the matrix solution. The sample plate is then analyzed on a MALDI hybrid triple quadrupole linear ion trap (QqQ_{LIT}) instrument equipped of a high repetition rate laser (*i.e.* up to 1 kHz). This MS platform offers unique capabilities to perform simultaneously a rapid screening and relative quantification in the selected reaction monitoring (SRM) mode, as well as sensitive MS/MS and MS³ experiments for confirmatory analyses.^{46, 112} In addition, the MALDI target can be analyzed in rastering mode at different speeds which has the advantage of recording the signal at each position alongside a single hair sample. Thus, hair MS imaging by MALDI-SRM/MS allows to monitor precisely drugs of abuse consumption history over the whole hair length. For confirmatory analyses, MS/MS experiments are performed using the enhanced product ion (EPI) scan mode, which consists in the fragmentation of precursor ion in the collision cell and the accumulation of fragments ions in the linear ion trap. Both EPI and MS³ experiments require a relatively long trap fill time of 300-500 ms in order to reach a sensitivity similar to the SRM mode.⁴⁶ This leads to an increased total scan time and a MALDI plate rastering at a lower speed than in SRM mode to acquire enough data points over the hair diameter. To avoid such long analysis time, the strategy is to perform targeted MS/MS and MS³ on a short distance (*i.e.* 2 mm), where the drugs were previously detected. In summary, the overall process from sample preparation to SRM relative quantification and MS/MS and MS³ confirmatory analyses takes approximately 2 hours, including the analysis of multiple intact hair samples. Finally it's worth mentioning that for qualitative investigations, only one intact hair sample is needed per measurement, while for relative quantitative measurements, several hair samples from the same strand are preferred. The remaining hair samples from the same individual can be stored and re-analyzed at a later stage, which is of importance in forensics when further investigations are requested.

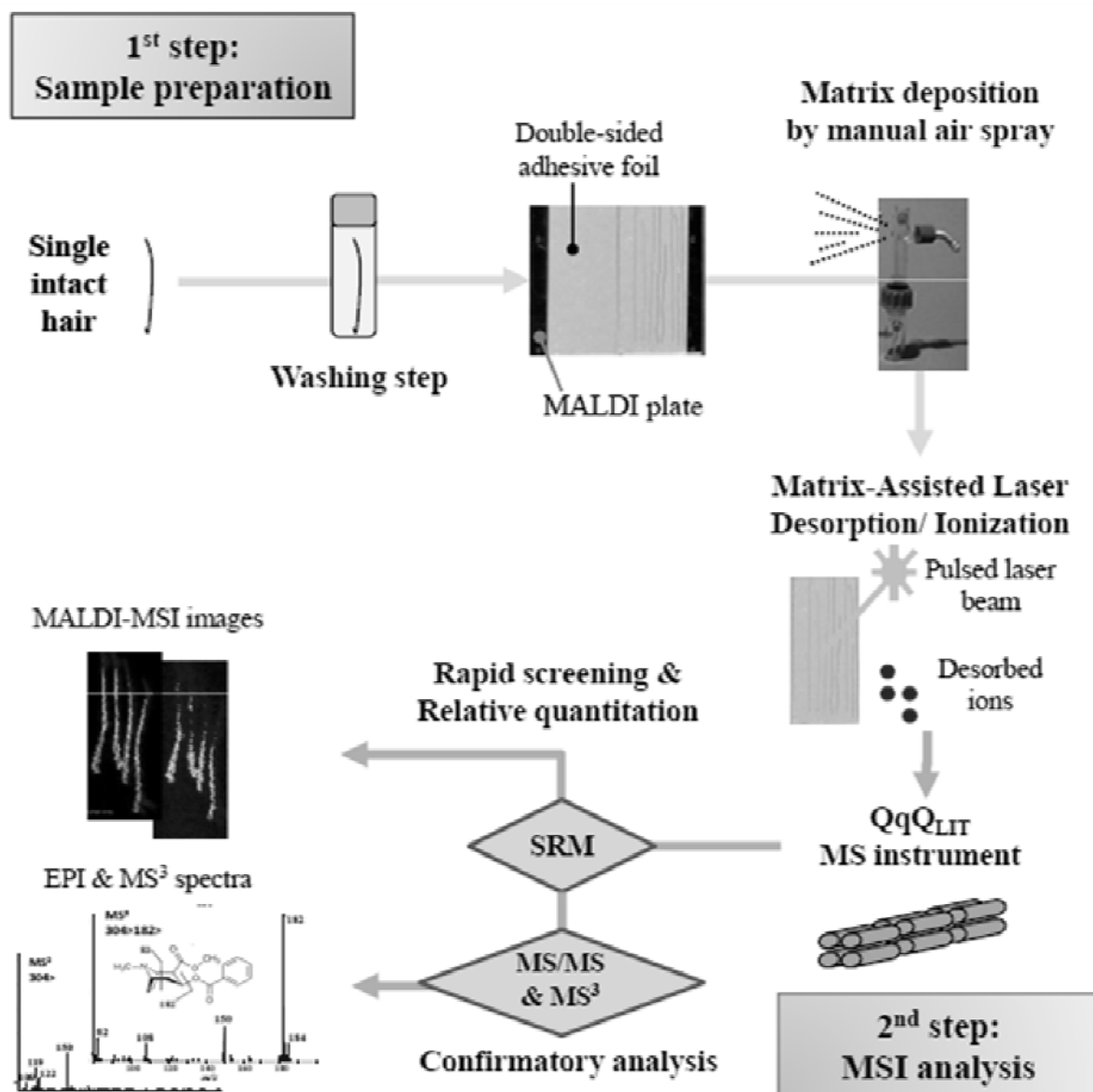


Figure III- 18. MALDI-MS analytical workflow for single hair analysis. The whole process from sample preparation to SRM relative quantification and MS/MS and MS³ confirmatory analyses takes approximately 2 hours, including the analysis of multiple samples.

3.4a.3.2. Detection and relative quantification of cocaine and its metabolites in single hair samples by MALDI-SRM/MS

The use of MALDI-SRM/MS for quantitative purpose has been extensively demonstrated in previous studies,^{39, 49-50} therefore it is applied herein to carry out relative quantification and characterize cocaine consumption over several months in a single hair analysis. The method was applied to quantitate cocaine and its metabolites in different hair samples available at the Institute of Legal Medicine of Zurich and already determined by routine LC-SRM/MS method. In the case of cocaine intake screening, MALDI has already shown adequate ionization and sensitivity for cocaine and its metabolites.¹⁰⁶⁻¹⁰⁸ MALDI-SRM/MS images of single hair samples have been acquired for monitoring the cocaine consumption from two subjects (Figure III- 19). Figure III- 19.a shows the optical image of a single hair from the individual H7 taped on the MALDI plate before applying manually the matrix by air spray.

Figure III- 19.b shows the MS images of five different intact hair samples from the same individual H7 where cocaine has been screened by MALDI-SRM/MS. All the five hair samples demonstrate similar cocaine consumption temporal information, where the drug concentration decreases from the hair tip to the scalp (*i.e.* 6-cm length segment) meaning that the individual consumption was probably reduced over the last 6 months. According to the recommendations of the Society of Hair Testing (SOHT)¹¹⁵, head hair is estimated to grow at approximately 10 mm per month. However, the correlation between drug spatial distribution in hair and the drug intake temporal information is not always straightforward to establish for several reasons such as i) the heterogeneous physiology of hair growth among individuals, ii) the alternative drug incorporation mechanisms,⁹⁷ iii) the influence of hygienic practices, iv) the occurrence of cocaine in hair of former drug users after a period of abstinence due to slow elimination kinetics of the drug.¹¹⁶⁻¹¹⁷ From the results shown in Figure III- 19.b,c it is obvious that MALDI-SRM/MS offers a much higher resolution towards drug spatial distribution than the segmental LC-SRM/MS analysis. If one would like to achieve a similar spatial resolution with the segmental hair testing approach, one would have to use shorter hair segments (*e.g.* 1-2 mm) which may be detrimental to the sensitivity due to the reduced amount of material available in each shorter segment.

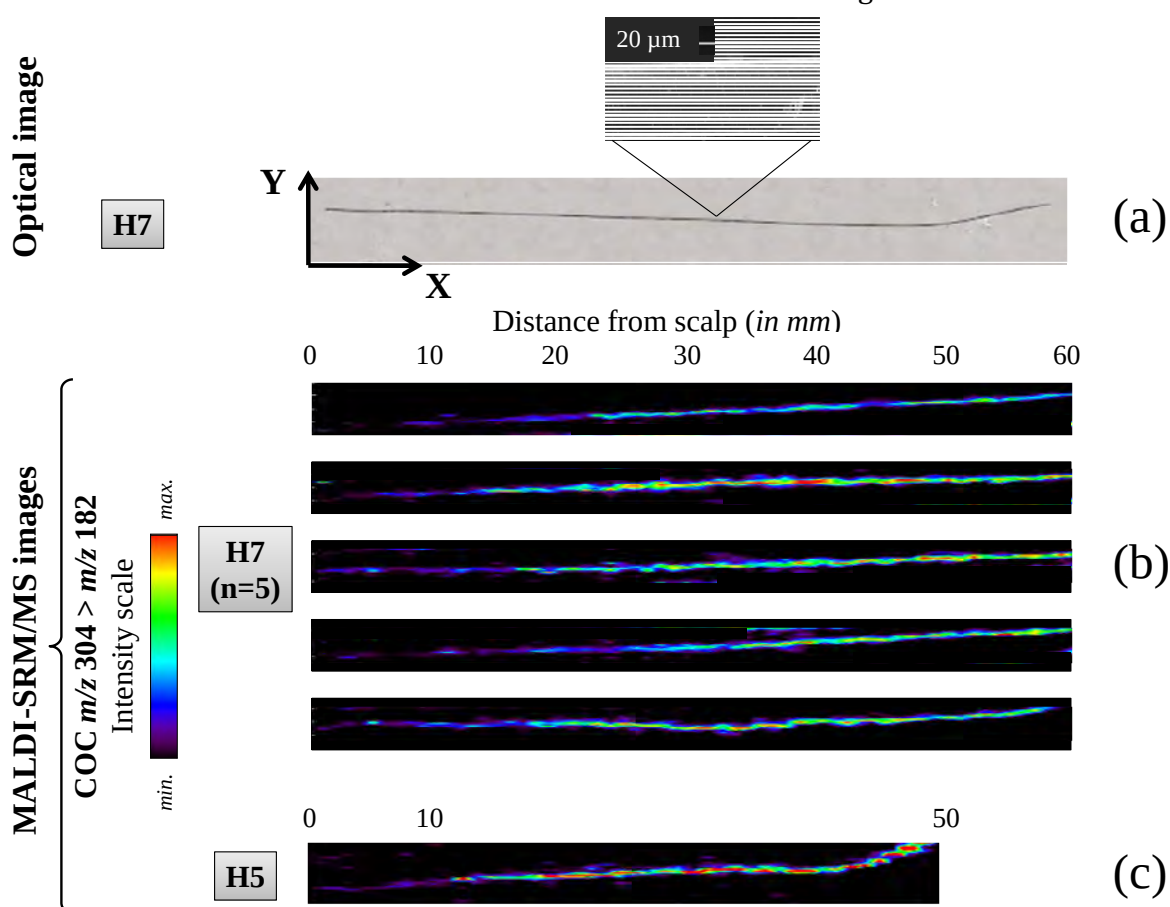


Figure III- 19. Imaging of cocaine along hair samples H7 and H5. (a) optical image of hair sample H7; MALDI-SRM/MS image based on the SRM trace of COC ($m/z\ 305 > m/z\ 182$) for (b) five replicates of single hair samples from individual H7 and (c) single hair analysis from individual H5. The quantitative results from LC-SRM/MS routine analysis were the following: 130 ng/mg (H7, whole sample); 4.9 ng/mg (H5, segment 0-10 mm) and 8.5 ng/mg (H5, segment 10-50 mm).

In order to assess the MALDI-SRM/MS reproducibility, each individual hair MS image was processed into segments of 10 mm-length (Figure III- 19.b – individual H7) and Figure III- 20 shows the relative quantification of cocaine and its metabolites in these segments. Each analyte signal was normalized by the signal of its respective deuterated standard to overcome inhomogeneous matrix spraying and the ratio was averaged for each 10 mm-length artificial segment. The average cocaine concentration found in each segment Figure III- 20.a) is well correlated with the decreasing concentrations of BZE (Figure III- 20.b) and ECOC (Figure III- 20.c) (individual values are given in supplementary Figure S1). However without chromatographic separation, direct MS analysis of isobaric metabolites (*i.e.* BZE and NCOC) may lead to under- or over-estimation of their measurement. Indeed, BZE and NCOC share the same precursor ion (m/z 290) and give the same most intense fragment (*i.e.* m/z 168) in collision-induced dissociation, leading to a metabolite cross-talk for the SRM transition m/z 290 > m/z 168. A solution is to monitor a second SRM transition specific for NCOC (*i.e.* m/z 290 > m/z 136) at the expense of sensitivity (Table III- 10). In our case, the contribution from NCOC in the SRM transition for BZE was found to be negligible for this subject.

Table III- 10. Signal/Noise comparison using CHCA and CI-CCA at 10 mg/ml. n.s. = not spotted.

Analyte name SRM transition	COC		ECOC		BZE				NCOC			
	304>182		318>196		290>168		290>105		290>168		290>136	
	CHCA	CI-CCA	CHCA	CI-CCA	CHCA	CI-CCA	CHCA	CI-CCA	CHCA	CI-CCA	CHCA	CI-CCA
Analyte amount (pg)												
150	n.s.	58	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
100	54	40	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
75	34	30	n.s.	n.s.	30	115	7	15	n.s.	n.s.	n.s.	n.s.
50	18	30	21	10	12	50	3	4	9	17	12	4
25	12	10	8	10	7	60	-	5	5	12	9	-
10	3	5	4	5	4	10	-	-	-	6	-	-
5	3	5	3	3	-	3	-	-	-	-	-	-

The observed relative standard deviation (RSD, n=5) is under 25 %, except for COC and BZE analysis of segment 20-30 mm where RSD is equal to 40 % and 30 % respectively. Although the five hair samples are from the same subject, the different hair growth phases might explain the variations observed among these hair samples. Indeed, the anagen phase is the active hair growth phase spanning over 2-3 years in which the incorporation of substances from the blood stream is taking place. Then, the catagen phase occurs for only 2-3 weeks as a transition state before the telogen phase which is spanning over another 2-6 months.¹¹⁸ It is obvious that the catagen and telogen hair growth phases do not represent the same time frame as the anagen phase but, unfortunately, differentiation of the different hair growth phases is not possible with cut hair since the root is missing. About 5 to 20% of head hair are in the telogen phase, leading to some kind of fuzziness in time correlation when analyzing hair strands or several single hair.

During the cocaine screening by MALDI-SRM/MS, detection of its metabolites (Scheme III- 4, Table III- 11) is required in the differentiation of its illicit consumption from an external contamination to prevent false positive results. Indeed, the SOHT recommends to quantitate

cocaine and at least one of the following metabolites: benzoylecgonine, ethylcocaine, norcocaine or ecgonine methyl ester.¹¹⁵

Therefore, additional SRM transitions corresponding to major metabolites formed during cocaine consumption were also monitored (*i.e.* benzoylecgonine (BZE), norcocaine (NCOC) and ethylcocaine (ECOC)). The MALDI-SRM/MS response factor ratios between the analytes were assessed by spotting standard solutions onto the adhesive foil at different concentrations (n=5). Thus, average response factor ratios were found to be 2 for COC/BZE, 0.6 for COC/ECOC and 8.5 for BZE/NCOC. In addition for the biological interpretation of the results, the BZE/COC ratio must be higher than 0.05 to conclude an endogenous cocaine incorporation.¹¹⁹ Based on the analyte MS response factor, all samples analyzed by MALDI-SRM/MS showed a signal ratio BZE/COC always superior to 0.05.

Finally, the limits of detection (LOD) that can be achieved by MALDI-SRM/MS were evaluated by analyzing hair samples with low cocaine and metabolites concentrations previously determined by the routine LC-SRM/MS method. When measuring the least concentrated hair samples by MALDI-SRM/MS, the lowest cocaine concentration detected was 4.9 ng/mg as illustrated in Figure III- 19.c for the first 10 mm of hair sample H5. Considering a hair diameter of 80 μm , a laser path of 100 μm and a hair density of 0.633 g/cm³ the maximum amount of analyte desorbed would be of about 20 fg which is within the performance of the MALDI-SRM/MS platform. This limit of detection permits to cover a wide range of case study, considering that the typical range described is 0.5-216 ng/mg¹²⁰ (0.1-33.7 ng/mg for BZE¹²⁰, 0.1-10.3 ng/mg for ECOC and 0.0-0.7 ng/mg for NCOC¹²¹).

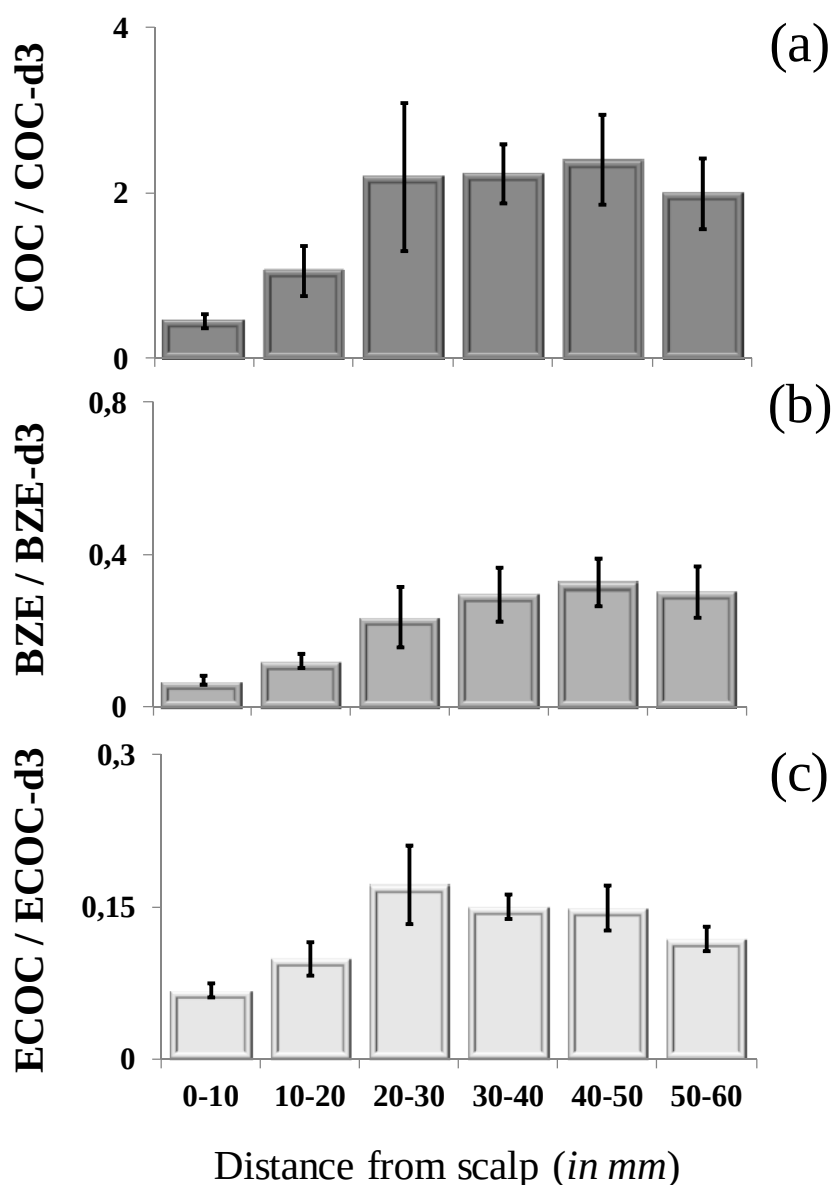
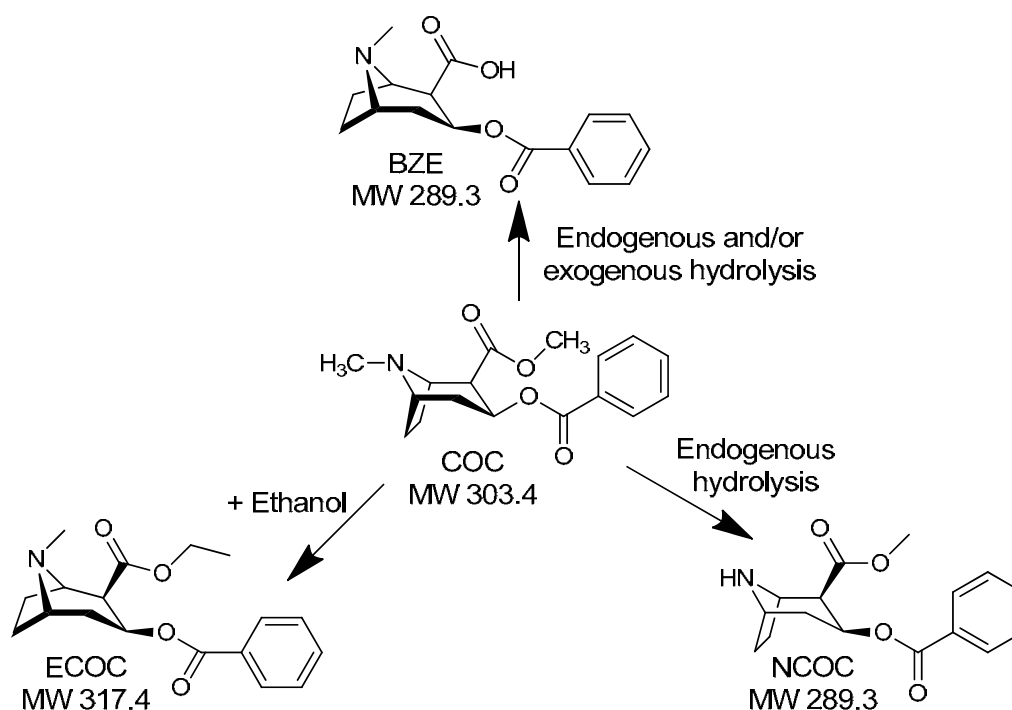


Figure III- 20. MALDI-SRM/MS relative quantification of cocaine and its metabolites along five single hair samples from individual H7. Histograms showing the evolution of (a) COC, (b) BZE and (c) ECOC concentration, after normalization of the signals with their respective deuterated standard (COC-d₃, BZE-d₃ and ECOC-d₃). The SRM transitions were: m/z 304 > m/z 182 for COC; m/z 307 > m/z 185 for COC-d₃, m/z 290 > m/z 168 for BZE; m/z 293 > m/z 171 for BZE-d₃; m/z 318 > m/z 196 for ECOC and m/z 321 > m/z 199 ECOC-d₃. The error bar represents one-fold the standard deviation around the mean (n = 5).



Scheme III- 4. Structures of cocaine and its investigated metabolites. COC: cocaine; BZE: benzoylecgonine; ECOC: ethylcocaine; NCOC: norcocaine; MW: molecular weight (in g/mol).

Table III- 11. Physico-chemical properties of cocaine and its main metabolites

Analyte	Formula	MW (g/mol)	pKa ^a	cLog P ^a	cLog D ^a pH ₃	cLog D ^a pH ₈
COC	C ₁₇ H ₂₁ NO ₄	303.35	8.97±0.60	3.08±0.38	-0.020	2.071
BZE	C ₁₆ H ₁₉ NO ₄	289.33	3.35±0.40 10.83±0.40	2.72±0.38	-0.004	0.214
ECOC	C ₁₈ H ₂₃ NO ₄	317.38	9.04±0.60	3.61±0.38	0.511	2.532
NCOC	C ₁₆ H ₁₉ NO ₄	289.33	9.16±0.60	2.14±0.38	-0.955	0.962

COC: cocaine; BZE: benzoylecgonine; ECOC: cocaethylene; NCOC: norcocaine; cLog P: calculated Log P;

cLog D: calculated Log D. ^a Calculated properties using ACD Labs Software v. 12.01.

3.4a.3.3. Confirmatory analysis based on MS/MS and MS³ experiments

MALDI-MS imaging is a direct analysis technique, since intact samples are analyzed without modification prior to its fixation onto the MALDI plate. Furthermore, because no chromatographic information is available with MALDI-MSI, structural confirmation of the analyte of interest is mandatory to complete the investigation and avoid false positive results. Two types of confirmatory MS experiments can be devised: a product ion experiment or an MS³ experiment using the linear ion trap mode of the QqQ_{LIT} instrument. To achieve good sensitivity, the trap fill time was set at 300 ms leading to a total scan time of ca. 2 seconds. This requires moving the plate at a lower speed (*ca.* 20-fold) than for the SRM quantitative experiments to permit to work at the hair scale resolution. In order to avoid such long analysis time, the strategy proposed here is to perform targeted MS/MS directly onto hair sample on a short distance (*i.e.* 3 mm), in a region where previous screening in the

SRM mode gave positive results for the substance of interest. Thereby, the MS/MS spectrum of precursor ion at m/z 304 (CE = 30 eV) allows the identification of the following cocaine fragment ions: m/z 108, m/z 119, m/z 122, m/z 150 and m/z 182 (Figure III- 21, Scheme III- 5). Since the fragment ions relative intensities are decisive to confirm the presence of cocaine in the sample, they were compared with those obtained from standard of cocaine spotted onto the MALDI plate. Results show that both MS/MS spectra from the standard spot and from the intact hair sample are in accordance.

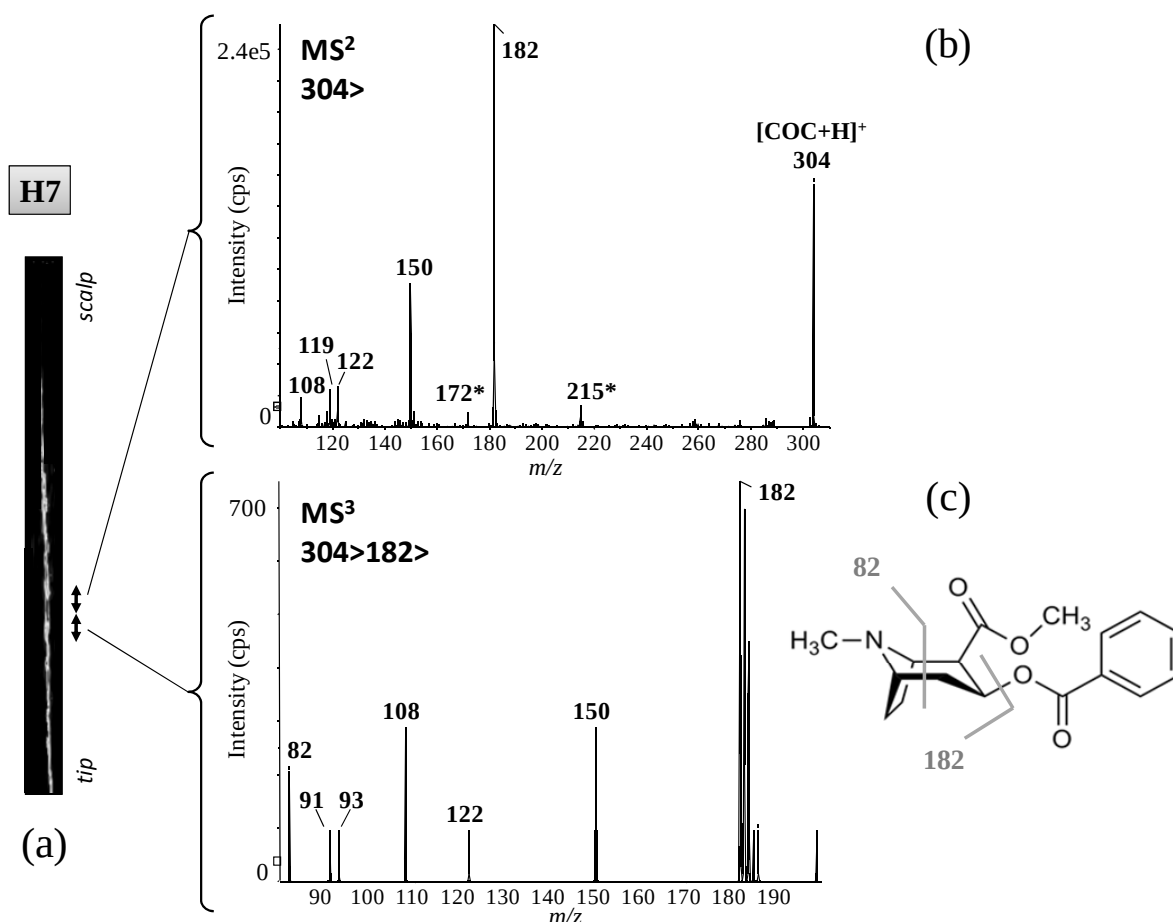


Figure III- 21. Confirmatory analyses of cocaine in intact hair sample H7. (a) MALDI-SRM/MS image based on the SRM trace of COC (m/z 304 > m/z 182) identifying regions of interest with high concentration of COC; (b) MS/MS (EPI) spectrum of [COC+H]⁺ at m/z 304 (CE = 30 eV), and (c) MS³ spectrum of m/z 304 > m/z 182 > from the regions of interest. * interfering ions from the CHCA-matrix.

Nevertheless, the MS/MS spectrum displays as well fragment ions at m/z 172 and m/z 215 (Figure III- 21.b). These fragment ions correspond to matrix-derived ions that are commonly observed in MALDI-MS/MS analysis, and which can interfere especially in the analysis of small molecules such as drugs of abuse. Therefore, selectivity can be improved by performing another confirmatory analysis such as an MS³ experiment.^{46, 112} Thus, the MS³ spectrum of m/z 304 → m/z 289 → displays only fragment ions from cocaine, *i.e.* m/z 108, m/z 122 and m/z 150 (Figure III- 21.c, Scheme III- 5), and the order of their relative abundances are in accordance with those observed in the reference spectrum (Table III- 12).

Table III- 12. Cocaine fragment ions intensity comparison observed in MS/MS (EPI) and MS³ experiments between a standard spot and an intact hair sample. The values represent the relative abundance compared to the base ion intensity at m/z 182 (100%). -: means that no signal was observed in the spectrum.

Fragment ion (m/z)		182	150	122	119	108	91
		Relative intensity (%)					
MS/MS	Standard	100	34	11	10	18	34
	Hair	100	36	11	9	8	-
MS ³	Standard	100	45	6	-	16	-
	Hair	100	42	24	-	39	-

3.4a.3.4. Experimental considerations for MALDI-MS imaging of a single hair sample

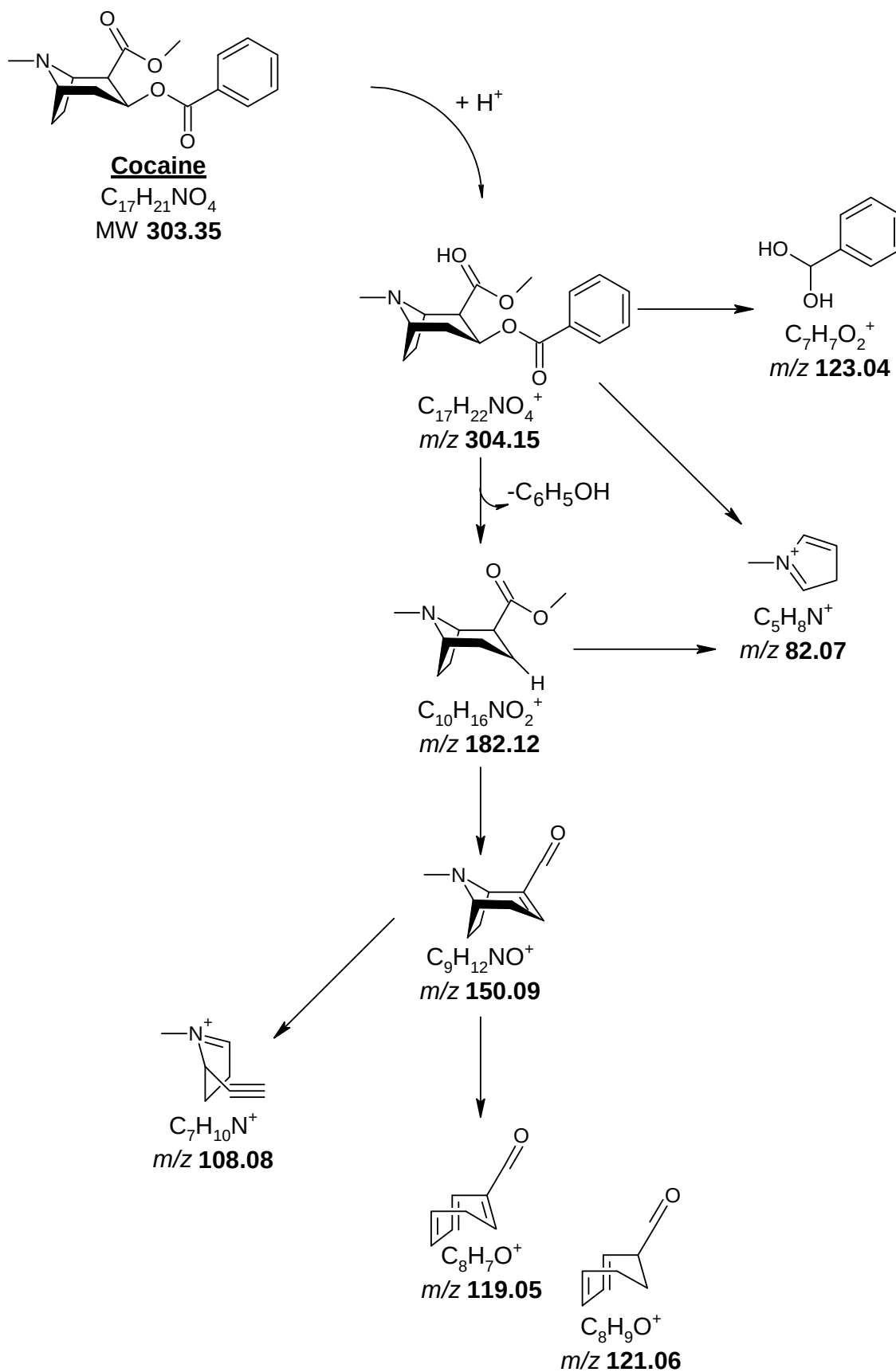
a) Spatial resolution

The MS images resolution is defined in two dimensions (see X- and Y-axes in Figure III- 19.a). The first dimension (*i.e.* X-axis resolution) corresponds to the distance between two adjacent laser paths across the hair perpendicular to its length. This dimension defines the X-resolution. Analysis time is increasing with smaller steps size. Due to the laser dimension constraint, the smallest X-step is of 0.1 mm corresponding to a theoretical average hair growth of about 8 hours. Steps of 1 mm were considered based on practical considerations in order to accelerate the MS image acquisition. Therefore, the analysis time of an intact hair sample of 6-cm length is approximately of 6 min in the SRM acquisition mode. Of course larger steps can be used to adjust spatial resolution depending on the toxicological question (*e.g.* single dose vs. regular consumption).

The second dimension (*i.e.* Y-axis resolution) depends on the plate speed and on the total duty cycle of the MS experiments. For a total duty cycle of 100 ms considering ten SRM transitions and plate speed of 1mm/s, the Y-resolution was of 100 μm , which is the minimum to acquire one pixel over the diameter of a single hair sample, assuming no radial diffusion.

b) Characterization of axial and radial diffusion

The MALDI-SRM/MS image of four segments of H7 hair sample is overlaid with a slight shift to the optical image (Figure III- 22.a). The COC signal is most intense in the middle of the SRM trace. By looking at a single raster SRM trace in the third segment, it was observed that the signal was recorded over ca. 800 μm (eight data points) in the Y-axis dimension (Figure III- 22.b). The diameter of H7 hair sample was determined by scanning electron microscopy and found to be of 80 μm . This suggests that the radial diffusion is quite significant. Table III- 13 shows the average radial diffusion found for segments 3 and 4 (calculated on $n=63$ raster lines – X-steps of 0.5 mm).



Scheme III- 5. General fragmentation pathway of protonated cocaine and interpretation of MS/MS and MS³ fragment ions.

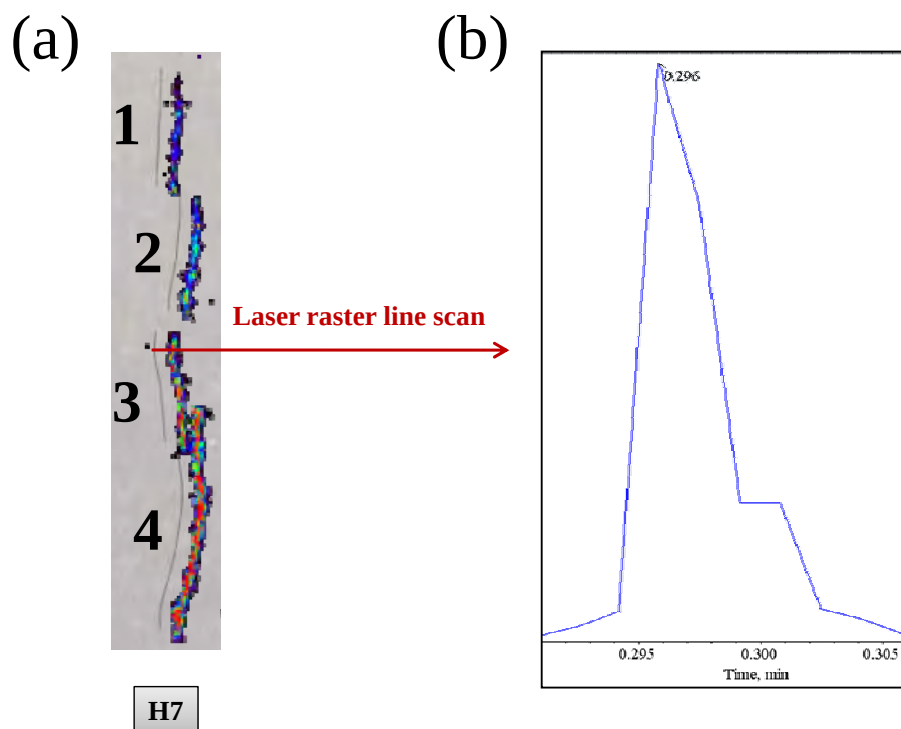


Figure III- 22. Characterization of axial and radial diffusions. (a) Hair sample H7 image of cocaine cut in four segments; and (b) SRM trace of cocaine in segment 3.

The axial diffusion (X-axis dimension) was also characterized to investigate if the chronological information is lost during MS analysis. For that purpose, the length of the hair segments was compared to the length of the corresponding SRM traces. The results are given in Table III- 14 and show an averaged variation of 0.6 mm (with a maximum of + 1 mm) compared to the measured lengths of hair segments.

Table III- 13. Characterization of the radial diffusion (segments 3-4)

Y-Resolution	100 μ m	
Width unit	min	mm
Mean	0.017	0.994
Standard deviation	0.003	0.198
RSD%	20%	20%
Number of points per SRM trace	10 $\pm$ 2	

Table III- 14. Characterization of the axial diffusion (segments 1-4)

Seg.#	Real size (mm)	Exp. Size (mm)	Variation	
1	10.7	11.6	+ 0.9 mm	+ 8 %
2	11.0	11.2	+ 0.2 mm	+ 2 %
3	10.0	11.0	+ 1.0 mm	+ 10 %
4	20.8	22.1	+ 0.3 mm	+1 %
Mean value			+ 0.6 mm	+5%

The radial diffusion may be explained in various ways: i) the Y-axis laser dimension being of 200 μm that is larger than hair diameter (80 μm), and considering that the analyte desorption may not be complete, it could be expected that the signal is measured over the 200 μm ; ii) the path of the laser through the sample can lead to a radial delocalization of matrix crystals containing the drug extracted from the hair. This has already been demonstrated¹²² and is revealed by the tailing peak shape of the SRM trace (Figure III- 22.b), and iii) a minor contribution might come from the solvent used to dissolve the matrix, which can delocalize the substance from its original position, leading to both radial and axial diffusion.

3.4a.4. Conclusion

A selective mass spectrometric approach based on MALDI-SRM/MS acquisition using a MALDI source mounted on a triple quadrupole linear ion trap has been developed to rapidly image cocaine and its metabolites distribution in a single hair sample. The low limit of detection was found to be in the range of 5 ng/mg. In addition, confirmatory analyses based on both product ion and MS³ experiments were performed to clearly identify the cocaine consumption. Using a high repetition laser with a laser beam width of 100 μm and a rastering step of 1 mm, it is possible to achieve a temporal resolution of hair growth of a few days. Since only part of the hair strand is analyzed, additional analyses are possible at any time on the remaining hair from the strand which is of importance in the field of forensic investigations.

3.4a.5. Supplemental material

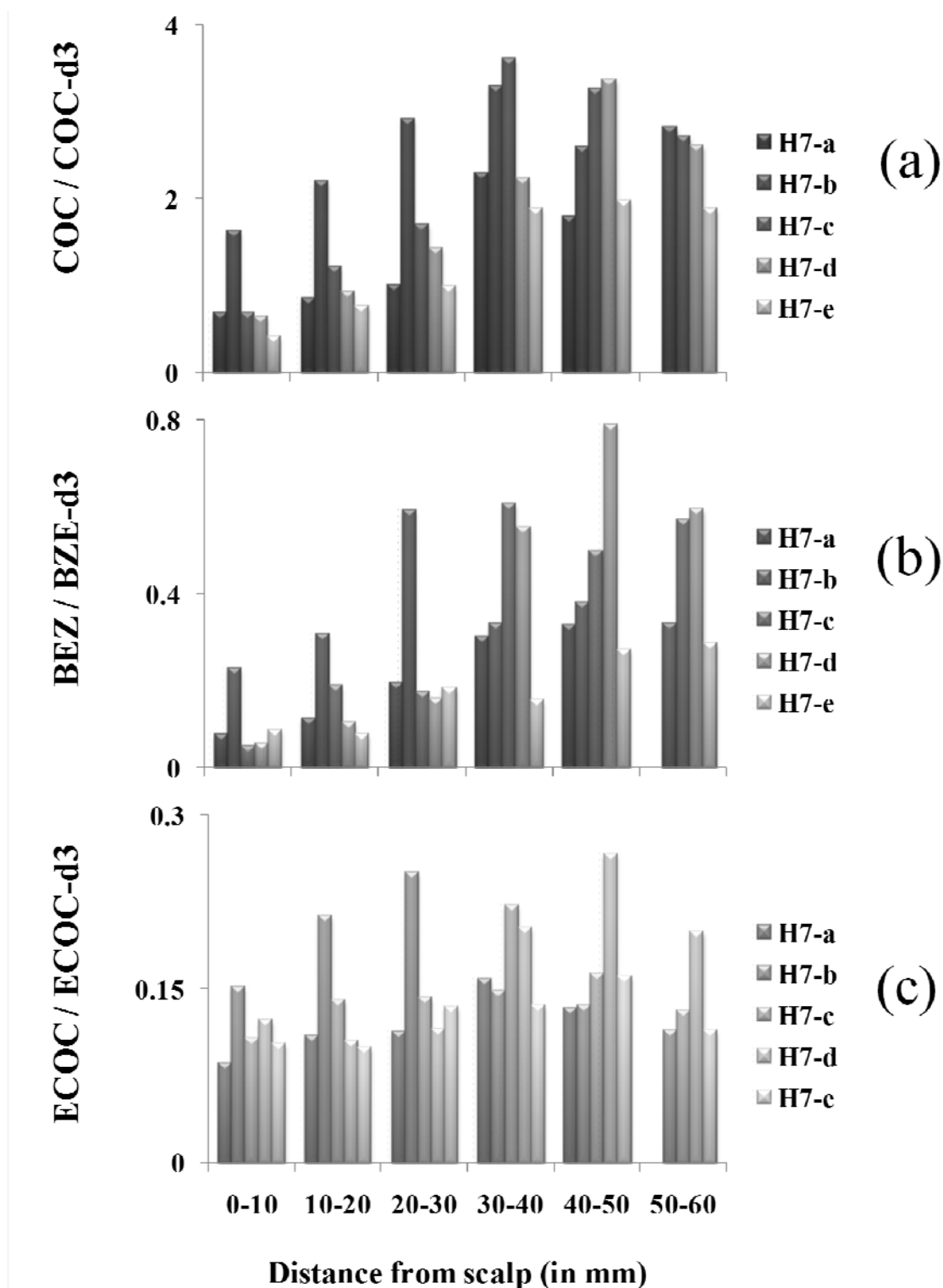


Figure S1. Histograms showing the evolution of (a) COC, (b) BZE and (c) ECOC, along 5 hair samples (H7-a to H7-e) belonging to the same individual (H7). The data are given after normalization of the signals with the signal given by deuterated compounds (COC-d3, BZE-d3 and ECOC-d3, respectively) to overcome inhomogeneous matrix spraying. The SRM transitions were: m/z 304 > m/z 182 for COC; m/z 307 > m/z 185 for COC-d3, m/z 290 > m/z 168 for BZE; m/z 293 > m/z 171 for BZE-d3; m/z 318 > m/z 196 for ECOC and m/z 321 > m/z 199 ECOC-d3.

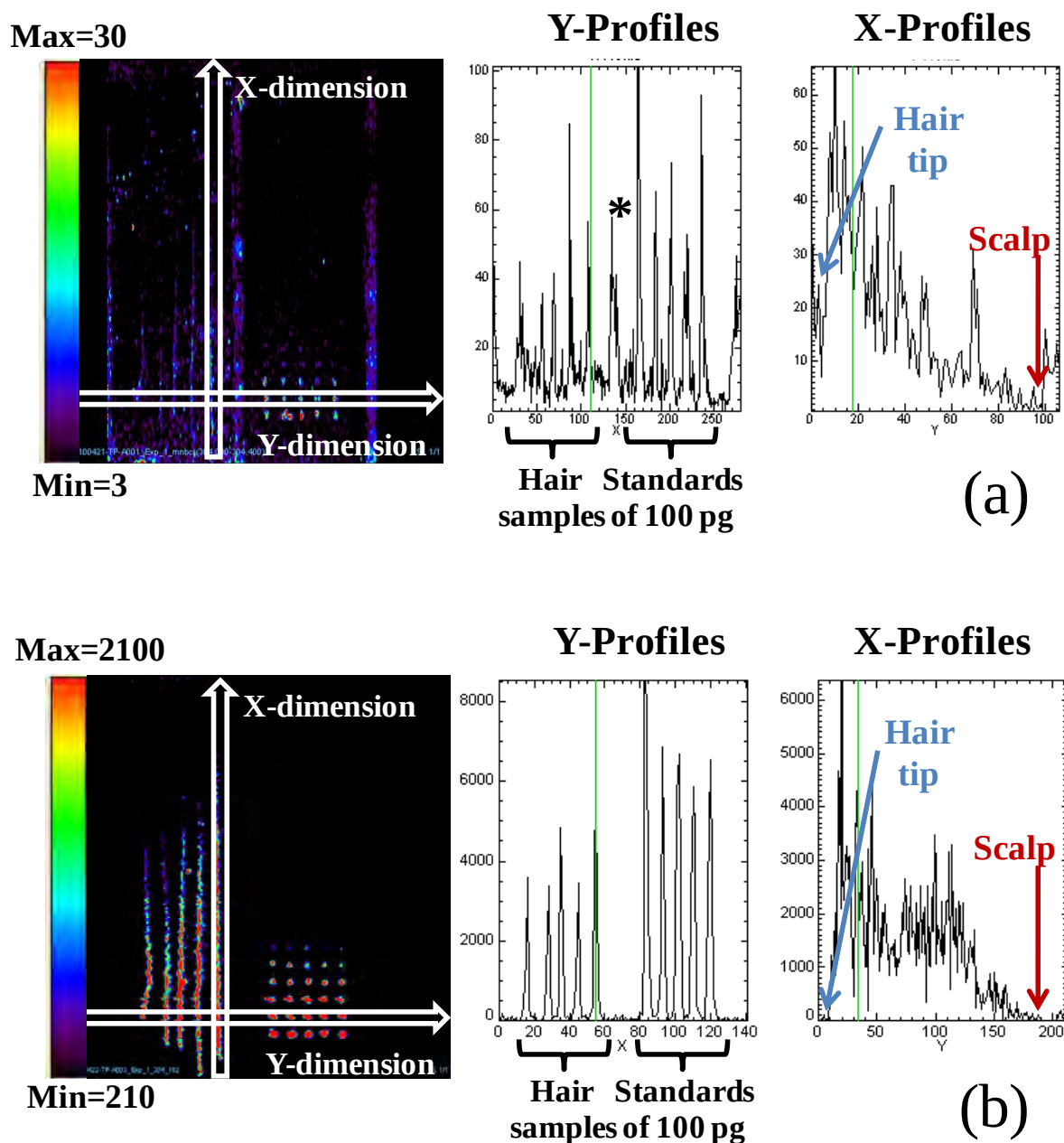


Figure S2. MALDI-MS(/MS) images corresponding to (a) full scan MS image based on the extracted protonated cocaine ion at m/z 304 and corresponding profiles along X- and Y-dimensions; and (b) SRM/MS image based on the SRM transition m/z 304 > m/z 182 of COC and X and Y profiles. Y-profiles show a signal decrease from the hair tip to the scalp.

*: intense signal from matrix in-between two adhesive foils.

Table S1. Analysis of 9 real samples using three different MALDI matrices: CHCA: 10 mg/mL; Cl-CCA: 10 mg/mL and NpCCA: 4 mg/mL.

Analyte name SRM transition	COC 304>182				BZE			
	CHCA	Cl-CCA	NpCCA		CHCA	Cl-CCA	NpCCA	
LC-SRM/MS (ng/mg)								
230								
170								
130								
99								
40								
6.70								
8.50								
4.90								
0.33								
0.26								

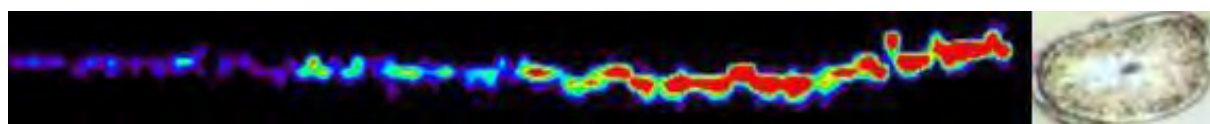
Analyte name SRM transition	NCOC 290>136				BZE			
	CHCA	Cl-CCA	NpCCA		CHCA	Cl-CCA	NpCCA	
LC-SRM/MS (ng/mg)								
44								
20								
13								
12								
12								
3								
0.75								
0.50								
0.11								
0.02								

Analyte name SRM transition	ECOC 318>196				NCOC 290>136			
	CHCA	Cl-CCA	NpCCA		CHCA	Cl-CCA	NpCCA	
LC-SRM/MS (ng/mg)								
12								
3								
1								
1								
0.38								
0.25								
0.02								

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

Part 4b – Multimodal analysis of cocaine in single hair sample: the contribution of high resolution ToF-SIMS imaging



Work in collaboration with:

Bryn Flinders, Ron M.A. Heeren (FOM Institute AMOLF, Amsterdam, The Netherlands)

Context of the study

This present section relies on unpublished data generated during the short term scientific mission (STSM) entitled “*ToF-SIMS imaging of drugs of abuse in single hair sample*”. This STSM was supported by the European Cooperation in Science and Technology COST action BM1104 (Reference: ECOST-STSM-BM1104-261112-024024) and hosted by Prof. Ron M.A. Heeren at the FOM Institute AMOLF (Amsterdam, The Netherlands). This work has been done in collaboration with Bryn Flinders, Gert B. Eikel (data analyst), Ivo Klinkert (software engineer), Anne Bruinen and András Kiss (assistance with ToF-SIMS mass spectrometer).

The main objective of the work carried out in the framework of this STSM was to support first investigations performed by MALDI mass spectrometry imaging (material published by Porta T. *et al.*⁴³) at the Life Sciences Mass Spectrometry laboratory (LSMS, Geneva, Switzerland) in order to address the following points:

- i) understand the diffusion observed by MALDI-MSI (i.e. comparison between matrix-enhanced SIMS and SIMS);
- ii) localization of the analytes (i.e. cocaine and metabolites) within the different layers of the hair (i.e. outer region [cuticle], cortex and medulla) by analyzing by high spatial resolution axial and longitudinal cross sections and determine if there is a axial diffusion at different time points (i.e. all along the hair sample).

3.4b.1. Introduction

Hair testing is a powerful tool routinely used for the detection of drugs of abuse in toxicology and forensic applications.¹⁰⁷ The analysis of hair is highly advantageous as it can provide prolonged detectability versus that in biological fluids and chronological information about drug intake based on the average growth of hair over several months. Current methodology routinely involves complex and time-consuming sample preparation (referred as segmental analysis) followed by gas or liquid chromatography coupled with mass spectrometry resulting in partial spatial loss, leading to a partial loss of the time information. One of the issues often raised in a legal context with this methodology is whether the drug is external contamination on the hair or present in the hair due to abuse.¹²³ To prove drug consumption and accumulation into hair, cocaine but also its metabolites have to be measured.

Preceded by a straightforward sample preparation, MALDI-MS imaging has been demonstrated as a suitable tool to image the distribution of cocaine, one of the most common used illegal drugs, as well as its metabolites in intact single hair samples from chronic users down to a concentration of 5 ng/mg.⁴³

It results from our first experiments that the MALDI-SRM/MSI platform we have developed suffered from two main limitations: i) the matrix manually applied by aerosol may delocalize the analytes; ii) the spatial resolution was of 100 μm due to instrumental constraints which prevent to perform complementary analysis of cross sections for instance to monitor axial and longitudinal distribution of the drug in hair. Taking into account that hair cross section may vary from 70 to 150 μm , improved resolution is almost mandatory to monitor axial and axial.

To go farther in our investigations, we wanted to answer the two following questions:

- What happens when applying a MALDI matrix on a hair sample?
- Answer biological question: what is the radial distribution of COC and metabolites within the different layers of a single hair sample?¹²⁴

Time-of-flight secondary ions mass spectrometry (ToF-SIMS) is a very interesting alternative to MALDI since it provides first information on the distribution of chemicals on surfaces without any chemical pre-treatments. Then, the focused ions beam enables to reach spatial resolution down to the μm -scale, and also allows in-depth profiling of the drugs of abuse incorporation in the different layers of a hair sample. This would enable to build a 3D-image to give the whole picture of the distribution of drugs of abuse and their metabolites in a single hair and over time by monitoring the signal all along the hair sample.

In addition, current decontamination protocols will be assessed by multi-modal mass spectrometry imaging in order to discriminate between external contamination or drug abuse consumption.

3.4b.2. Experimental section

3.4b.2.1. Sample preparation

a) Chemicals

Cocaine (COC), benzoylecgonine (BZE) and ethyl-cocaine (ECOC) were provided by Lipomed (Arlesheim, Switzerland). Norcocaine (NCOC) was provided by Cerilliant (Molsheim, France). Methanol (MeOH, HPLC grade) was provided by Sigma-Aldrich (Buchs, Switzerland). α -cyano-4-hydroxycinnamic acid (CHCA), carboxymethylcellulose (CMC) and trehalose were provided by Fluka (Buchs, Switzerland). Formic acid (HCOOH) was purchased from Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

b) Washing protocol

Prior analysis, single hair samples were washed in order to avoid any external contamination which may come from sebum, sweat, substance powder, etc. and may lead to false positive results. The washing protocol consisted in washing in water, acetone, and finally hexane (each step: 30 s shaking, 20 mL) as described in previously published material,⁴³ following an adapted protocol used for routine analysis of hair strands at the Institute of Legal Medicine of Zurich. After drying at room temperature, the intact washed hair samples were either embedded for further sectioning or fixed onto stainless steel plates or ITO glass slides using different supports as specified in the corresponding parts.

c) Sample preparation for the analysis of intact hair samples

After being washed, the intact samples are mounted onto conductive indium tin oxide (ITO) glass slides (resistance of 4-8 Ω (Delta Technologies, Stillwater, MN, USA)) or stainless steel plate by using conductive tapes (carbon, copper); nonconductive double sided adhesive tape; glue, conductive carbon cement.

d) Sample preparation for the analysis of axial and longitudinal sections

Several embedding material have been used and prepared as follow: i) ultrapure water sonicated for 16 minutes to remove gas; ii) gelatin: solution at 10% was prepared by dissolving 5 g in 50 mL of water; iii) carboxymethylcellulose (CMC): solutions at 1% (1 g in 100 mL of water) or 2% (2 g in 100 mL of water) were prepared by adding the CMC powder in pure water and stored at 40°C for 1 day to obtain viscous liquid; iv) trehalose: solution at 10% was prepared by dissolving 10 g in 100 mL of water and was stored at +4°C between uses.

Hair samples were embedded in different polymers and snap frozen afterwards using liquid nitrogen for cross-section analysis. The frozen block was placed in the sample holder with water. The embedded hair samples were then sectioned at a thickness of 12 μm using a Microm HM550 cryo-microtome (Microm International, Walldorf, Germany, chamber temperature = -20°C) and immediately thaw-mounted onto the sample holder (ITO glass

slide or stainless steel plate when specified). Samples were then stored at -80°C prior to the analysis.

According to the medical dictionary (Stedman's) (<http://www.drugs.com/dict/>), an axial section referred to a “cross section obtained by slicing [...] the body or any part of the body structure, in a horizontal plane, a plane that intersects the longitudinal axis at a right angle. Synonym = transverse section”; a longitudinal section is a “cross section obtained by slicing in any plane parallel to the long or vertical axis [...] the body or any part of the body or anatomic structure”.

e) Contamination of hair samples

In order to assess the effectiveness of the washing protocol, hair samples were contaminated by immersion in a solution of cocaine at a concentration of 1 µg/mL (overnight incubation).

3.4b.2.2. MALDI-ion mobility mass spectrometry

MALDI-ion mobility acquisitions have been performed on a Waters MALDI HDMS SYNAPT mass spectrometer (Waters Corporation, Manchester, UK). Experimental conditions were: laser energy: 200 (200 Hz); trap collision energy: 20 eV; mass range: m/z 50-350; spatial resolution of the image generated: 50 µm x 150 µm.

3.4b.2.3. ToF-SIMS imaging

ToF-SIMS mass spectrometry imaging experiments have been performed on a Physical Electronics TRIFT II TOF-SIMS mass analyzer (Physical Electronics, USA) equipped with a gold liquid metal ion gun tuned for 22keV Au⁺ primary ions. The extractor current is maintained between 2.25 and 2.38 V during the analysis.

3.4b.2.4. LAESI mass spectrometry

Laser-ablation electrospray ionization (LAESI) mass spectrometry has been performed on a LTQ Velos mass spectrometer (Magnet of 7T, Thermo Scientific) equipped with LAESI DP-1000 Direct Ionization System (Protea Biosciences). MS data were acquired with the AGC off, an injection time of 1 ms and 1 microscan for a total MS scan time of about 90 ms with a mass range between m/z 150 and 2000. For the LAESI-MS analysis a laser center-to-center spot distance of 400 µm was used with 10 laser pulses at each location. In addition, a laser frequency of 10 Hz with a laser energy of about 700 mJ with a laser spot size of 200 µm and an output wavelength of 2.94 µm was used. The ESI solution was 1:1 MeOH:H₂O with 0.1% acetic acid, applied at a flow rate of 1.0 µL/minute and an ESI voltage of 4 kV. The ESI emitter (New Objective) was a 5 cm stainless steel emitter with a 100 µm ID and a 320 µm OD. The samples were analyzed with no additional sample preparation at ambient temperature and pressure. LAESI Desktop Software (LDS) in conjunction with Xcalibur was used for data acquisition.

3.4b.3. Results and discussion

3.4b.3.1. MALDI –(ion mobility) – time-of-flight mass spectrometry

Initially standards of cocaine (COC) and benzoylecgonine (BZE) were analysed by MALDI-MS and MS/MS, the MS analysis showed the presence of cocaine $[M+H]^+$ at m/z 304 and benzoylecgonine $[M+H]^+$ at m/z 290. The MS/MS analysis of both compounds showed the presence of an abundant product ion at m/z 182 for cocaine and m/z 168 for benzoylecgonine.

Intact single hair samples were coated with CHCA and analysed using MALDI-MS/MS imaging. The images showed the distribution of the product ion at m/z 182, derived from the precursor ion of cocaine at m/z 304 (Figure III- 23). As an example, the distribution of cocaine all along the hair sample H7, following the distribution of the product ion at m/z 182, displays a higher concentration in close to the tip than the scalp region, which correlates the description of the same sample by T. Porta *et al.*⁴³ The results correlate well to previously published data using the MALDI-QqQ_{LIT} platform operated in the selected reaction monitoring detection mode.⁴³

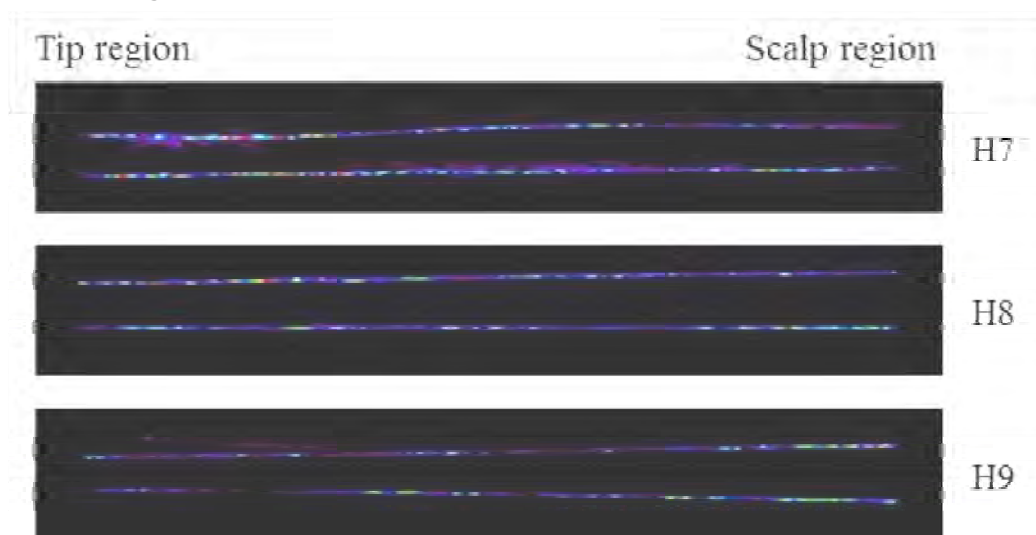


Figure III- 23. MALDI-MS/MS images of the distribution of the product ion at m/z 182, derived from the precursor ion of cocaine at m/z 304 within hair samples. Acquisitions were performed on the MALDI-SYNAPT HDMS mass analyzer. Pixel size = 50x150 μ m

One of the limitations of direct analysis in general is to suffer from the lack of chromatographic separation prior to MS detection, which is particularly critical for the analysis isomeric compounds. Cocaine for instance has two isomeric metabolites, benzoylecgonine (BZE) and norcocaine (NCOC), which have the same molecular weight and thus the same exact mass for the protonated ion. Tandem mass spectrometry (MS/MS) is required to provide selectivity, but also ion mobility subsequent to the surface sampling could be envisaged as an alternative. Unfortunately, the first results obtained with the MALDI SYNAPT HDMS mass analyzer operated in the ion mobility mode do not allow for the separation of the two isomeric BZE and NCOC (Figure III- 24).

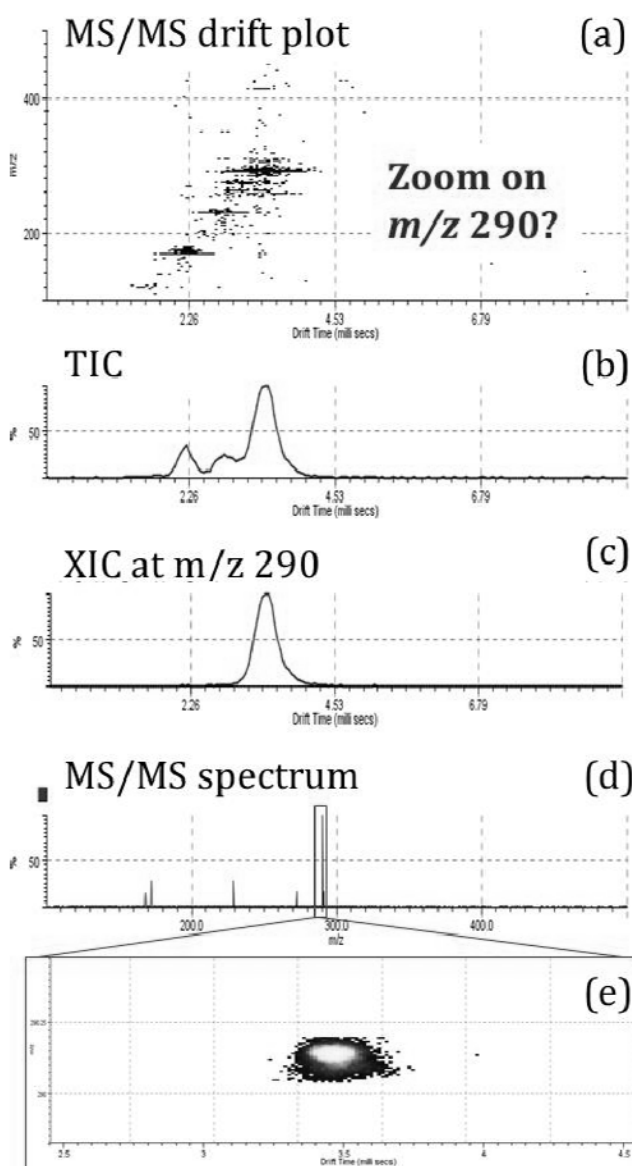


Figure III- 24. MALDI-IMS-ToF-MS/MS analysis of a standard mixture of benzoylecgonine and norcocaine.

(a) MS/MS drift plot;

(b) Total ion count from ToF-MS/MS acquisition;

(c) XIC of the ion at m/z 290 showing a single peak with a drift time of 3.4 ms;

(d) MS/MS spectrum for the precursor ion at m/z 290 (collision energy = 20 eV) averaged over 3.2 – 3.5 ms;

(e) Zoom of the drift blot centered on the mass region $290 < m/z < 290.25$.

3.4b.3.2. ToF-SIMS mass spectrometry imaging of single intact hair samples

Unwashed and washed samples containing cocaine have been analyzed and its results that the washing step is mandatory not only to remove the external contamination of the sample but also to remove chemicals. In a regular 2D experiment, only the upper few nanometers of the sample surface are sampled and chemicals such as PDMS (polydimethylsiloxane, which is the most widely used polymer based on silicon and used in shampoos for shiny and slippery properties) localized on the surface of the sample hamper the quality of the SIMS analysis by leading ionization suppression. Washed intact samples have been then mounted onto ITO glass slides using conductive carbon or copper tapes, either with and without CHCA coated on the surface of the sample but nor cocaine or one of its metabolites could be detected. The same results are obtained without and with gold coating prior to the ToF-SIMS analysis. Figure III- 25 displays the ToF-SIMS image of a single washed hair sample mounted onto conductive carbon tape after gold coating: the TIC recorded at the surface is homogeneously distributed on the surface of the sample but cocaine could not be detected (Figure III- 25.b,

spectrum Figure III- 26.a). It results from all these experiments that COC could not be detected on hair surface.

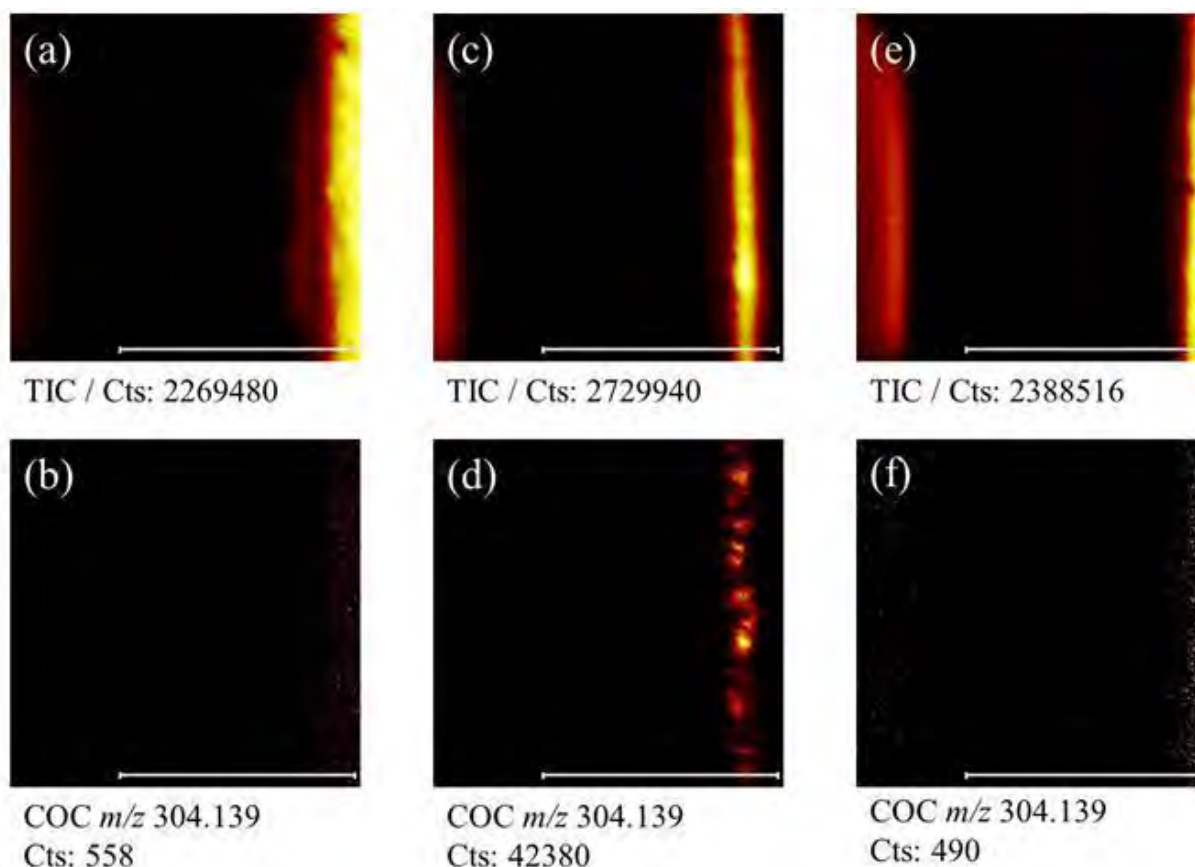


Figure III- 25. ToF-SIMS images resulting from the analysis of (a,b) control hair sample (washed); contaminated samples (c,d) unwashed and (e,f) after washing following the standard protocol. (a,c,e) Total ion current; (b,d,f) extracted ion for [COC+H]⁺ at m/z 304.139 corresponding to the protonated ion of cocaine. The samples have been gold coated prior to the analysis. Scale bars = 100 μm . Color bar: minimum intensity = black; maximum intensity = yellow.

3.4b.3.3. Assessment of decontamination procedures on the analysis of drugs in hair samples

In order to assess the effectiveness of the washing protocol hair samples were contaminated by immersion in a 500 μL of a solution of cocaine at a concentration of 1 $\mu\text{g}/\text{mL}$ (overnight incubation). When analyzed by TOF-SIMS without washing the results clearly showed a strong signal for cocaine ([M+H]⁺) at m/z 304.139 (Figure III- 25.d, spectrum Figure III- 26.b). Following washing cocaine was no longer detected on the surface of the hair sample (Figure III- 25.f, spectrum Figure III- 26.c). This means that the hair surface is free from cocaine and the washing step prior the analysis enables to remove potential external contamination.

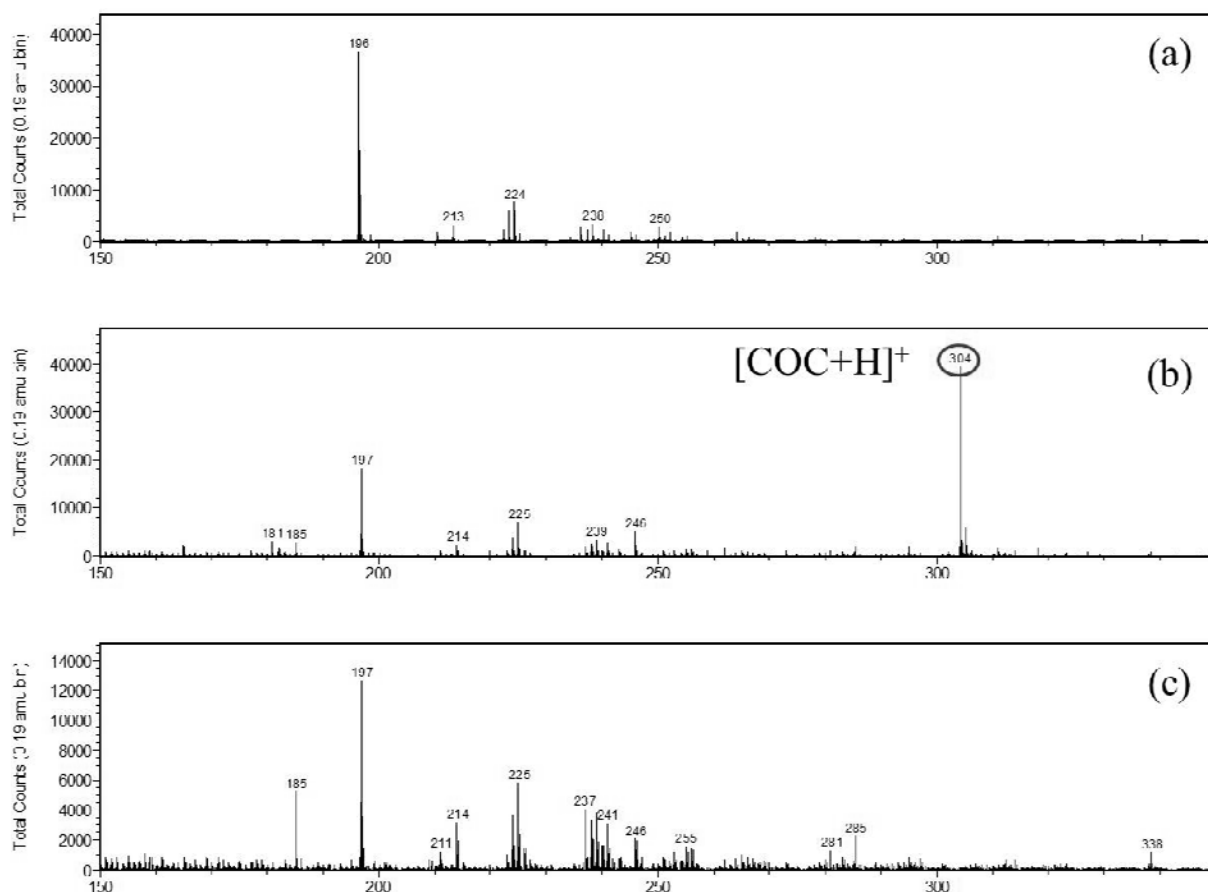


Figure III- 26. MS spectra from (a) control hair sample (washed); contaminated samples (b) unwashed and (c) after washing following the standard protocol.

One of the first objectives of the study was to compare the results obtained from SIMS and ME-SIMS after CHCA coating, in order to better understand the lateral diffusion of the analyte that have been described by T. Porta *et al.*⁴³ However, since cocaine could not be detected in intact hair by SIMS (even with Au coating), the comparison is not possible. One complementary analysis would be to analyze a contaminated unwashed sample and to coat its surface by applying a MALDI matrix. However, the round shape of intact hair makes its analysis by ToF-SIMS challenging as it leads to the "shadowing" effect from the primary ion beam. The resulted image does not reflect the real shape of the "object" as the secondary ion beam is deflected. Complementary analyses of axial and longitudinal sections, which have a flat shape, are required.

3.4b.3.4. Sample preparation for sectioning hair samples

The sectioning of hair in the axial and longitudinal axes remains by far the most challenging step of the sample preparation. Manually cutting the hair samples longitudinally is very difficult, and specialized holder would be needed.

The strategy developed in the laboratory consists in embedding a single hair sample. The most challenging part is to maintain the hair straight within the polymerized block and the material required here is quite straightforward, easily available in any analytical laboratory and consists only in small magnets and plastic tubes (Figure III- 27.a). The first step requires

clipping each of the extremities of a single hair sample (Figure III- 27.b). The sample is then plunged into the plastic tube and stays straight (Figure III- 27.c), filled with the embedding material beforehand.

The plastic tubes are then flash frozen in liquid nitrogen and stored at -80°C prior to the next step of the sample preparation. Axial sections of 12 µm-thickness thaw-mounted onto the sample holder (ITO glass slide or stainless steel plate when specified) are then analyzed by ToF-SIMS imaging.

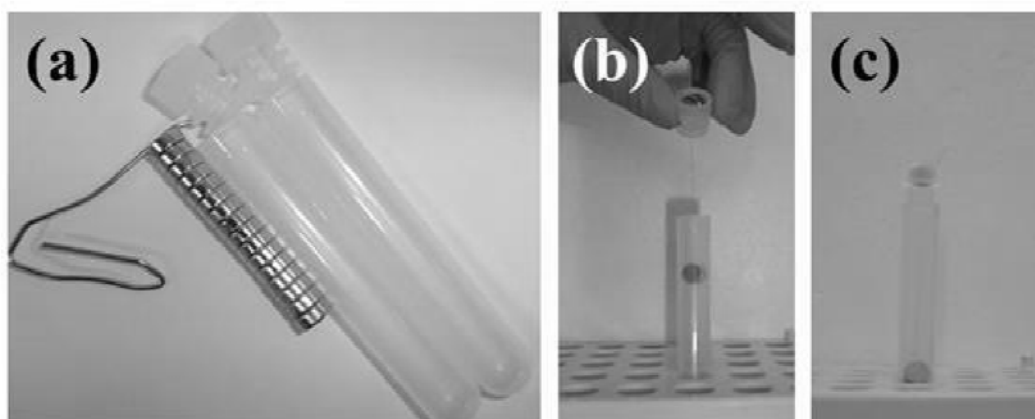


Figure III- 27. Pictures showing: (a) material required for the embedding of intact hair sample; (b) single hair clipped between magnets; (c) straight hair in plastic tube filled with embedding material.

3.4b.3.5. ToF-SIMS imaging of single hair samples

a) Imaging cocaine distribution in axial section of hair by ToF-SIMS: preliminary results obtained after embedding in gelatin 10%

Axial cross-sections are analyzed using ToF-SIMS after embedding in gelatin 10%. Results show the presence of cocaine (Figure III- 28.b) as well as from the metabolite associated with the simultaneous consumption of alcohol and cocaine, i.e. ethylcocaine at m/z 318 (Figure III- 28.c). The high resolution images generated (i.e. pixel size of 1.5 x 1.5 µm) enable to localize the two compound mainly in the cortex region of the hair but not in the medulla, which is indicative of uptake.

The signal of the two compounds in the medulla is equal to the signal of the same ions displayed in a region out of the section, while the signal in the cortex is above 3 times the chemical background.

This contrast between the medulla and the cortex regions is pointed out Figure III- 29 showing the profiles of cocaine in axial section after embedding in gelatin 10% along several axes.

These new results validate the washing protocol showing its efficiency to remove potential external contamination, without extracting the analytes from the deeper layers of the hair.

From these analyses and the optical image, it results that the hair section displays a flattened shape instead of a round shape. This might be due to the soft properties of the gelatin used as embedding polymer for the cryosectioning process.

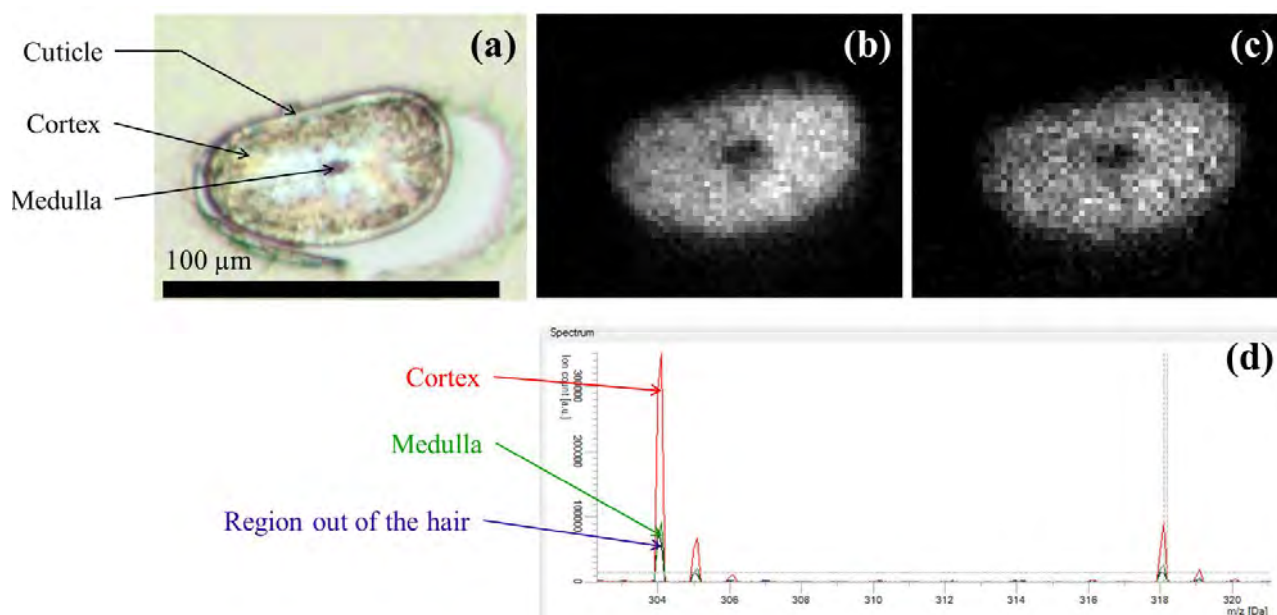


Figure III- 28. (a) optical image of a axial section obtained after embedding the hair sample in gelatin 10%; ToF-SIMS images based on the (b) protonated ion of cocaine (m/z 304.1-304.2) and (c) protonated ion of ethylcocaine (m/z 318.1-318.2); (d) mass spectra (m/z 303 to 321) showing the signal of the targeted ions from the different layers of the hair sections (i.e. cortex, red; medulla, green) and from a region out of the section (blue trace). The images are generating using Data Cube Explorer. The pixel size is of 1.5 µm.

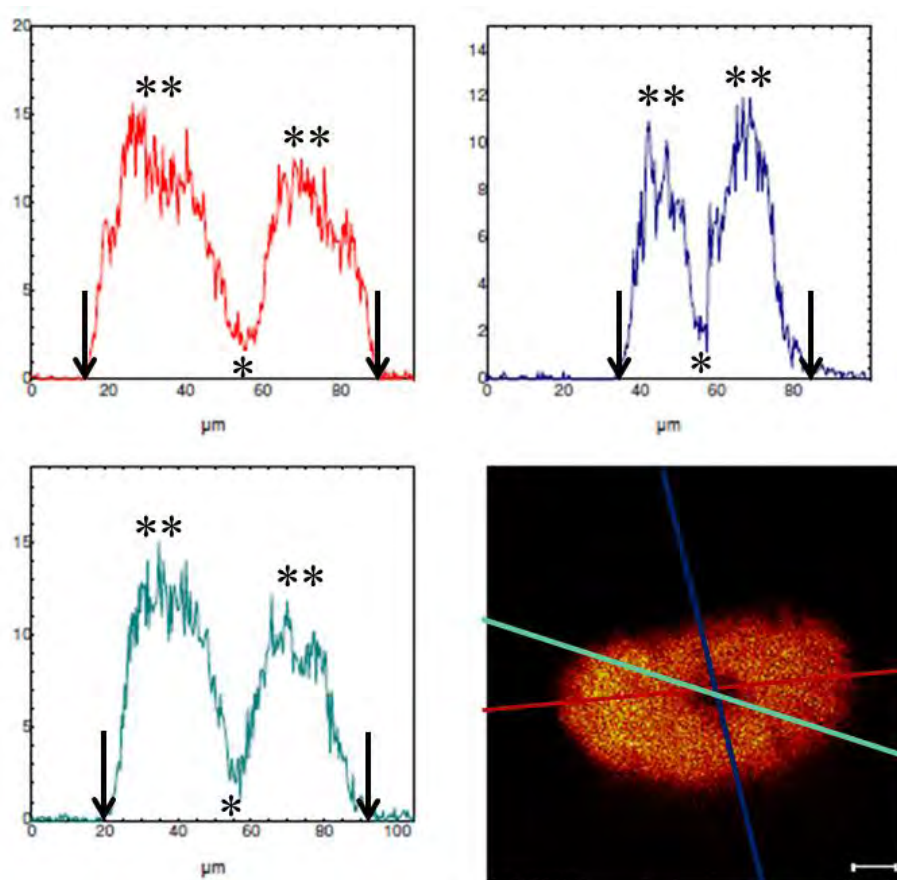


Figure III- 29. Profiles of cocaine in axial section after embedding in gelatin 10% along several axis. **: cortex region. *: medulla region. The arrows indicate the outer borders of the section. Scale bar = 10 µm.

b) Evaluation of different embedding material for cryosectioning of axial sections

Several materials are evaluated as embedding material. The optical images are shown Figure III- 30. The sections prepared after embedding in trehalose, prepared at a concentration of 10%, also display a flattened shape. Moreover, during the freezing step, some crystals appeared and are observable on the surface of the section as shown (Figure III- 30.b). Carboxymethylcellulose (CMC) is also employed as embedding material. Due to its low solubility in water, only 1% and 2% solutions are prepared, resulting in high viscous liquid and a hard polymer. The image with CMC 2% shows that the round shape is maintained (Figure III- 30.d).

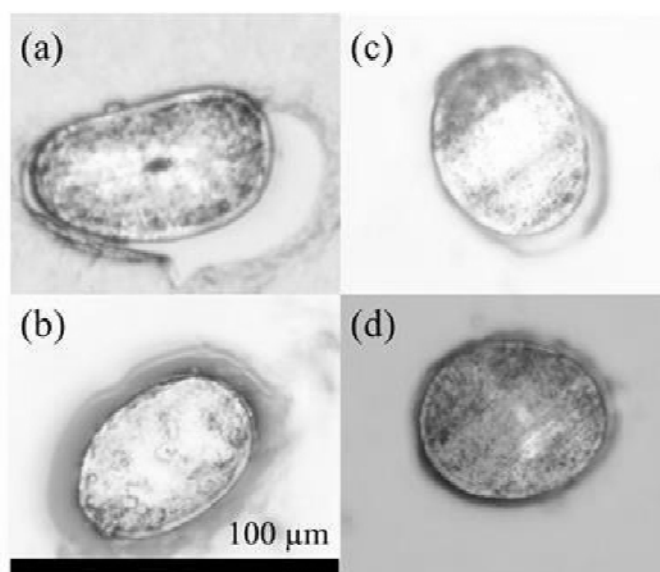


Figure III- 30. Optical images of a axial section (10 μ m) cut after embedding the hair sample in: (a) gelatin 10%; (b) trehalose 10%; (c) CMC 1% and (d) CMC 2%.

Therefore, CMC 2% is used for further investigations. However, it is worth noting that some cautions have to be taken in the handling of the embedded samples.

c) Cryosectioning of hair in the longitudinal axis

By analyzing longitudinal sections, one goal is to monitor the consumption of drugs of abuse all along a single hair sample by ToF-SIMS. This part of the sample preparation is by far the most challenging. First, the sample has to be very straight in the embedding polymer and also precisely parallel to the edge of the cryotome, to cut the whole specimen and not only part, which very is challenging to achieve manually with high precision. In addition, during the development of a protocol to cut longitudinally a hair, it appeared that the hair sample does not stuck to the polymerized block and is lost during the cutting. The same phenomenon occurred as well as with gelatin than with CMC.

As complementary analysis, stronger material has been envisaged (e.g. acrylic Technovit resin¹²⁵). The strategy consists in embedding the sample in a strong block, then to polish it and finally analyze the whole block by ToF-SIMS. However, here again, Technovit was not convenient to handle and polishing was found to damage the sample.

Additional optimization is on-going at the Institute AMOLF to perform these longitudinal sections by using a specific plate with different hole at defined depth (Figure III- 31), as previously described by Kempson *et al.*¹²⁶ The method is expected to produce relatively smooth, planar samples ideal for further analysis with surface sensitive techniques for the investigation of internal distributions of elemental and molecular species.

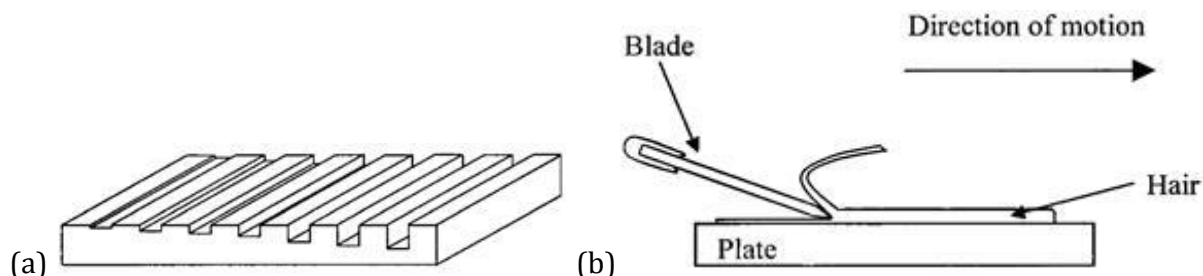


Figure III- 31. (a) Schematic of the plate with hole of 10 to 80 μm deep (60 mm long). (b) Longitudinal section of hair sample obtained by cutting to the desired depth with a blade. From ref.¹²⁶

d) Cocaine monitoring in axial sections by ToF-SIMS imaging

Alternatively to the longitudinal sections analysis, the cocaine consumption can be monitored from axial sections cut at different time points as illustrated Figure III- 32. This would also allow for the determination of the axial distribution all along the sample and if diffusion occurs over the time, which is of prime of importance from a biological point of view and for forensic investigations.

After the primary analysis, it results that no differences could be observed. Figure III- 32 displays the distribution of cocaine all along the hair sample. The overall distribution (given by the average of 3 adjacent sections for a single time point) exhibits the same pattern than previously published and validated results, i.e. cocaine is detected at an increasing concentration from the scalp region (= the sampling region) to the tip of the hair sample.⁴³

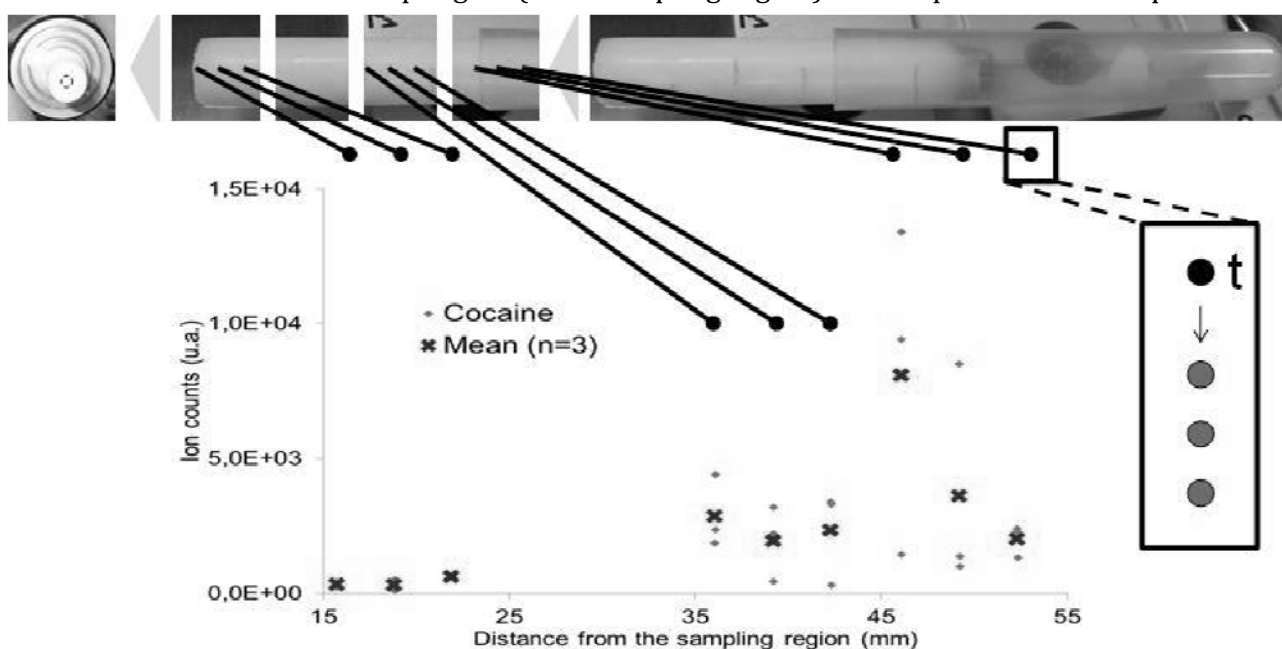


Figure III- 32. Monitoring cocaine in axial sections cut all along the hair sample (sample H7⁴³).

Although the sample preparation of hair sectioning in the axial dimension is now well controlled in our lab, this procedure is time consuming and a high variability in between two adjacent sections is observed. This is partly due to the complex incorporation mechanism of drugs into hair. The model proposed is complex and states drugs and metabolites are incorporated into hair during formation of the hair shaft (via diffusion from blood to the actively growing follicle), after formation (via secretions of the apocrine and sebaceous glands), and after hair has emerged from the skin (from the external environment). Further, drugs can be transferred to hair from multiple body compartments or pools located in tissues surrounding the hair follicle. It's noteworthy that these mechanisms could be drug-specific and also concentration dependant.¹²⁷ Inter- but also intra-individual heterogeneity may also lead to this high variability in the signal detected.

In addition, we were concerned about the link between the spatial information obtained, which is guided by the analytical strategy employed for sampling, and its significance from a biological point of view. Figure III- 33 shows that the area sampled with the 100 μm -width laser, horizontally rastered over the intact hair sample, represents approximately 7-10 h of hair growth (assuming a growth of 1 cm / month), which is 10.5X to 15X the cocaine half-life time (i.e. 40 minutes in human). On the other hand, by using SIMS as surface sampling technique of axial tissue sections of 12 μm -thickness each, 1 h of the hair growth is image, and even less because only the first nm of the surface are sampled with SIMS. Results show that sampling a wider window in the time scale minimize the variability

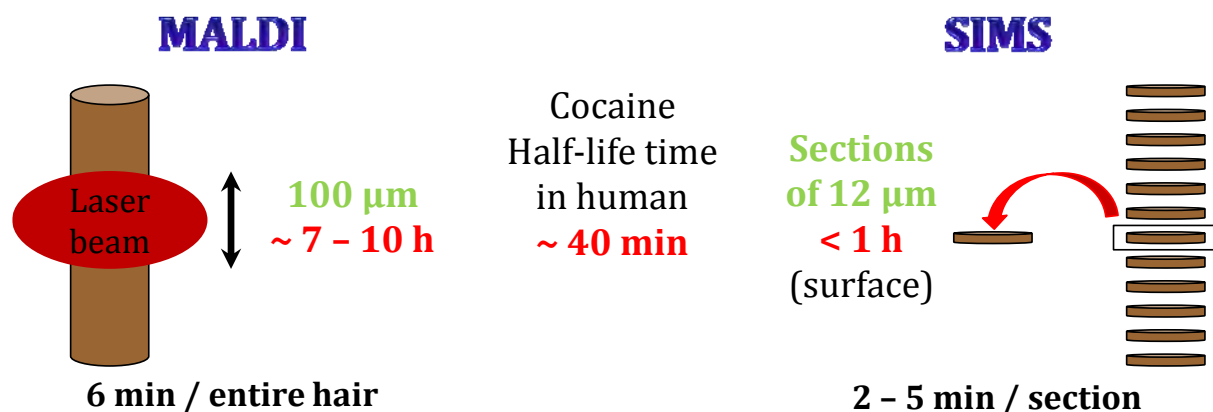


Figure III- 33. Comparison between the spatial information conditioned by the analytical technique employed and the corresponding time scale, considering a hair growth at ca. 10 mm/month.

3.4b.3.6. LAESI mass spectrometry

Complementary analyses were performed by laser-ablation electrospray ionization (LAESI) mass spectrometry (hyphenation: LTQ-FT-ICR). The preliminary results do not enable to detect cocaine and its metabolites in a single intact hair sample (washed). Interestingly, after the analysis, the surface of the hair sample remains intact and no injuries caused by the laser, even at the maximum power energy (Figure III- 34). Further instrumentation optimizations are on-going such as: varying of the ablation time per discrete point sampled, composition of the solvent used for the ESI.

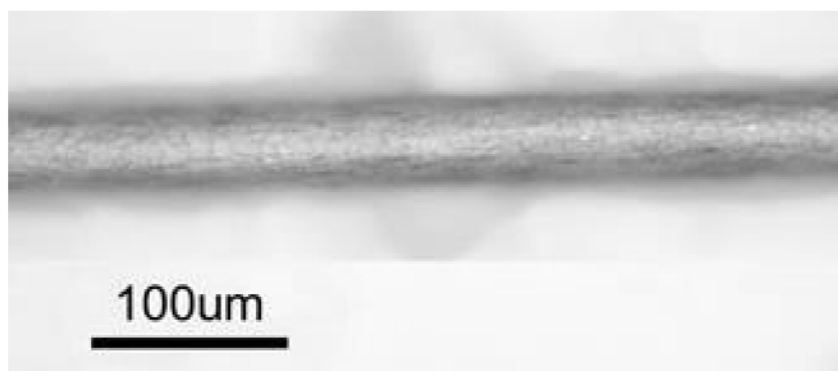


Figure III- 34. Optical image of a hair after LAESI-MS analysis. The distance between 2 discrete points is of 200 µm

The damages caused by the Nd:YAG laser has also been evaluated in our lab using scanning electron microscopy (SEM) and has also revealed no damage at the surface of the hair after laser path. The mechanism explaining why drugs of abuse can be detected by MALDI mass spectrometry without any sample has not been determined so far (e.g. do the scales forming the medulla, the outer layer of the hair composed by keratin, are being opened at the same time than the laser hit the surface of the hair? And do they recover their initial position after the analysis, explaining why SEM microscopy revealed no damage?). The most probable hypothesis is that the analytes are extracted from the inner layer of the hair during the MALDI matrix application.

The multimodal approach developed proves the effectiveness of our washing protocol. It also confirms cocaine does not come from possible external contamination (i.e. cocaine and its metabolite still detected in the axial sections by Tof-SIMS after the washing step).

3.4b.4. Conclusions and perspectives

During this collaborative work, an extensive effort has been focused on the sample preparation. A straightforward and inexpensive strategy has been developed and is now well controlled to perform axial cryo-sections of single hair samples.

This study shows the complementarity between MALDI and SIMS ionizations to study single hair samples. The MALDI-QqQ_{LIT} platform has been proven particularly suited for the fast screening and time course of cocaine and its metabolites on intact hair samples. ToF-SIMS imaging offers the main advantage to work without applying a matrix, which is one of the most critical step of the sample preparation preceding the MALDI-MS imaging experiments. Due to its high resolution capability, HR-ToF-SIMS allows for the localization of cocaine and its metabolites ethylcocaine within the different layers of the hair. Results show the two compounds mainly distributed in a homogenous way within the cortex region.

HR-ToF-SIMS is a valuable tool to answer biological questions and is expected to be used in support to further investigations for a more precise understanding of the mechanisms involved in the incorporation of drugs into hair. This is critical for forensic scientists in order to interpret the results of hair analysis properly.

Some points haven't been addressed yet and further investigations will be conducted in order to:

- Improve the sample preparation for the sectioning of longitudinal sections as described above;
- Perform 3D-ToF-SIMS imaging in depth profiling mode to establish a 3D-image of the drug within the sample;
- Although it was not possible to characterize the diffusion occurring on intact hair sample after matrix application, additional experiments will aim at characterizing the lateral diffusion on axial sections (i.e. also by comparison between SIMS *versus* ME-SIMS after matrix coating).

CHAPTER 3.

Application of MALDI-SRM/MS imaging to plant biology and forensics

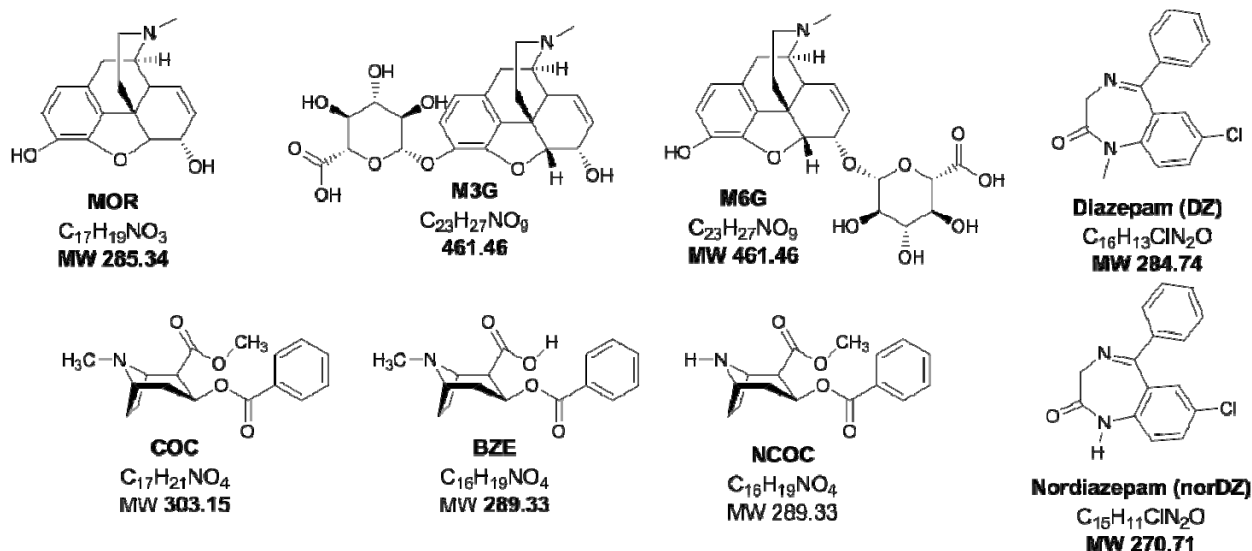
Part 5 – Multimodal analysis of drugs of abuse in postmortem human tissues by MALDI-MS imaging and LESA-DMS-MS/MS from the same tissue section

3.5.1. Introduction

To date, mass spectrometry coupled with liquid or gas chromatography has largely been employed in forensics to analyze drugs of abuse in various matrices (e.g. oral fluid, plasma, blood, urine as well as hair¹²⁸⁻¹³⁰). Sometimes, when common biomaterials are not available, post-mortem analysis of human tissues is required for the investigation of drugs of abuse. Many approaches have been successfully developed, mainly based on liquid or gas chromatography, but the analysis of such complex biomatrices usually requires several steps for sample preparation. These include organ dissection, homogenization, and specific extraction. The objective of this study is to present a straightforward direct tissue analysis using mass spectrometric imaging (MSI). Although in the past MSI has been mainly focused on proteins/peptides analysis¹³¹ and the distribution of pharmaceuticals and metabolites in rodents,^{45, 132-133} MSI can be a very powerful tool for forensic investigation. MALDI-MSI does not require any complex sample preparation from tissue cutting to matrix coating prior to MS analyses. In contrast with other methods which require several extraction steps from homogenized samples (and thus which need a larger amount of material), the major advantages of MALDI-MSI are: (i) a straightforward sample preparation prior to MS analysis, (ii) no loss of analyte spatial distribution, (iii) a small sample consumption (i.e. only μm -thick slices needed) for sensitive analysis and the possibility of storing the sample and re-analyzing it at a later stage (i.e. samples are kept intact as little material is ablated at the surface of the slice).

The method development will focus on the screening and quantification of morphine (MOR), diazepam (DZ), cocaine (COC), and their respective metabolites (i.e. morphine glucuronide (MG), including the two forms morphine-3-glucuronide M3G and/or morphine-6-glucuronide M6G; nordiazepam (NorDZ); benzoylecgonine (BZE) and norcocaine (NCOC), respectively; see Scheme III- 6).

This part also introduces a new analytical platform dedicated to the analysis of isomeric metabolites, based on differential ion mobility spectrometry (DMS) operated after a direct surface sampling based on liquid extraction (i.e. LESA, liquid extraction surface analysis).



Scheme III- 6. Structures of drugs of abuse and metabolites investigated

3.5.2. Experimental section

3.5.2.1. Chemicals

Morphine (MOR), morphine-6-glucuronide (MG), morphine-d₃ (MOR-d₃), diazepam (DZ), nordiazepam (norDZ), diazepam-d₅ (DZ-d₅), cocaine (COC), benzoylecgonine (BZE) and cocaine-d₃ (COC-d₃) were provided by Lipomed (Arlesheim, Switzerland). Methanol (MeOH, HPLC grade), and acetonitrile (ACN, HPLC grade) were provided by VWR International (Nyon, Switzerland). Formic acid (HCOOH) and trifluoroacetic acid (TFA) were provided by Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

3.5.2.2. Immunoassays

Ablated kidneys from dead bodies were directly ice-frozen and stored at -80°C prior to the analysis. Immunoassays (CEDIA homogeneous enzyme immunoassay) were performed as routine analyses for opiates and benzodiazepines. Because urine was not available, a dialysate of kidney tissue was used after enzymatic hydrolysis for the immunoassays. Quantitative analyses from blood were performed using GC-MS for opiates and/or GC-ECD for benzodiazepines (analysis performed by the Institute of Legal medicine of Zurich, Switzerland) and are given Table III- 15.

Table III- 15. Immunoassays and quantitative GC-MS and HPLC-MS analysis of postmortem human tissues

Sample	Immunoassay	Method for quantification	Concentration in peripheral blood (ng/ml)
A	Negative	-	-
B	Negative	HPLC-MS for opiates GC-ECD for benzodiazepines	Morphine: 80 MG: not quantified Diazepam: 140 Nordiazepam: 210
C	Positive	GC-MS	Cocaine: 8800 Benzoylecgonine: 8900
D	Positive	GC-MS	Cocaine: 6 Benzoylecgonine: 3800

3.5.2.3. Tissue sections preparation

Frozen samples were brought up to -20°C and cut at *ca.* 12 µm thickness using a cryotome (Leica, Leica Biosystems) and immediately thawed-mounted onto stainless steel plate. For MALDI-MSI experiments, α-cyano-4-hydroxycinnamic acid (CHCA) and 4-chloro-α-cyanocinnamic acid (Cl-CCA) were used as matrices at a concentration of 10 mg/mL in appropriate solvent mixture. For the investigation of the best MALDI matrix, 200 nL-droplets of CHCA and Cl-CCA matrix solutions (10 mg/mL in acetonitrile/water/TFA, 50:50:0.1, v/v/v) were manually spotted in and out of the kidney region. For relative

quantification of drugs, *c.a.* 3 mL of CHCA at 10 mg/mL was applied to each small MALDI plate 44 x 44 mm by aerospray at a distance of 20 cm. The pressure of the nebulizer gas was kept constant at 0.2 bar during the whole process.

3.5.2.4. MALDI-MS imaging experiments

MS images were performed on a prototype MALDI triple quadrupole linear ion trap (MALDI 4000 QTRAP) fitted with a high repetition rate laser (Nd:YAG laser 355 nm, 1000 Hz, energy value of 2.0 μ J) operating in a horizontal rastering mode. Acquisitions were performed in positive ion ionization mode using both enhanced product ion (EPI) scan and selected reaction monitoring (SRM) experiments. For the EPI experiments, the plate was moved at a rastering speed of about 0.25 mm/s and 8 scans were summed (scan rate 1000 amu/s, total scan analysis = 4 s, compatible with the plate speed to get an image resolution of 1 mm). For the SRM experiments, the plate speed was 2 mm/s and the total scan analysis was 250 ms (image resolution of 500 μ m). The SRM transitions monitored were: m/z 286 > m/z 201 for MG (CE = 35 eV; DP = 90 V); m/z 462 > m/z 286 for MG (CE = 35 eV; DP = 90 V); m/z 285 > m/z 154 for DZ (CE = 38 eV; DP = 90 V); m/z 271 > m/z 140 for NorDZ (CE = 40 eV; DP = 80 V); m/z 304 > m/z 182 for COC (CE = 27 eV; DP = 70 V); m/z 290 > m/z 168 for BZE/NCOC (CE = 26 eV; DP = 70 V) (i.e. transition #1, common to the two metabolites); m/z 290 > m/z 136 for NCOC (CE = 26 eV; DP = 70 V) (i.e. transition #2, specific to NCOC) and m/z 307 > m/z 185 for COC- d_3 (CE = 27 eV; DP = 70 V).

3.5.2.5. Liquid extraction surface analysis (LESA) conditions

Surface sampling was performed using a chip-based nanoelectrospray (nES) system (TriVersa NanoMate, Advion BioSciences). Conditions were: aspirated solvent: 2 μ L, dispensed solvent: 1 μ L, post-aspirated solvent: 1.5 μ L (after 10 seconds). Extraction was performed with MeOH/H₂O (80/20, v/v) + 0.1% HCOOH. A 0.4 psi gas pressure (nitrogen) and a 1.55 kV voltage were applied to the sample to generate the nES.

3.5.2.6. Differential ion mobility spectrometry (DMS) mass spectrometry

Experiments were performed in positive ion ionization mode on a Q TRAP 5500 mass spectrometer (AB Sciex) equipped with a prototype DMS cell in front of the orifice plate. ACN, MeOH, EtOH, toluene and acetone were evaluated as DMS modifiers and introduced in the cell using a pump system (Agilent 1100) to obtain 1.5% in 10 psi curtain gas. DMS operating conditions were: temperature = 150 °C (250 °C for toluene); separation voltage = 4'000 V (3'500 V for toluene). Information dependent acquisition (IDA), based on a survey scan of the compensation voltage (from - 40 V to 10 V) in SRM mode, and dependent experiments performed in MS/MS and MS³ modes, were found to be beneficial in improving the selectivity and enable the simultaneous screening and relative quantification of drugs of abuse in tissue sections.

3.5.2.7. Software for data acquisition and processing

The MALDI source and its laser were controlled using custom M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and data collection. PeakView software (v.1.0, AB Sciex) was used for raw data processing (.wiff files). A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files (.wiff format) into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex) used for MS images generation and processing. ChipSoft Manager software (v.8.3.2.1209, Advion BioSciences) was used to control automatic liquid extraction from surface sample with the TriVersa NanoMate. A dedicated driver provided by Advion BioSciences was used to create and launch the batches.

3.5.3. Results and discussion

3.5.3.1. Workflow for direct surface analysis approaches

While several steps are needed prior to LC-MS or GC-MS analyses, direct analysis is particularly attractive for the forensic investigation of drugs of abuse in postmortem human tissue sections, because they require little to no sample preparation. Direct analysis involved the analysis of flat samples. For this purpose, frozen tissue samples (i.e. kidneys) are cut into thin sections (i.e. 12 μm) using a cryotome and the sections are then thaw-mounted on either stainless steel plates or glass slides, depending on the required application. The tissue sections can then be: i) stored at -80°C prior to analysis or ii) directly analyzed. The LESA approach does not require further sample preparation (Figure III- 35, upper part). MALDI requires the coating of a matrix. In the present study, the MALDI matrix, i.e. CHCA dissolved at 10 mg/mL in an acidified organic solution, is applied by manual airspray with a TLC sprayer (Figure III- 35, lower part). The coating step lasts from 30 to 40 minutes depending on the matrix solution. The sample plate is then analyzed and both platforms are mounted on hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass analyzers. The merits of this platform have already been discussed several times in this Chapter (Part 1, Part 4a) and will not be detailed here.

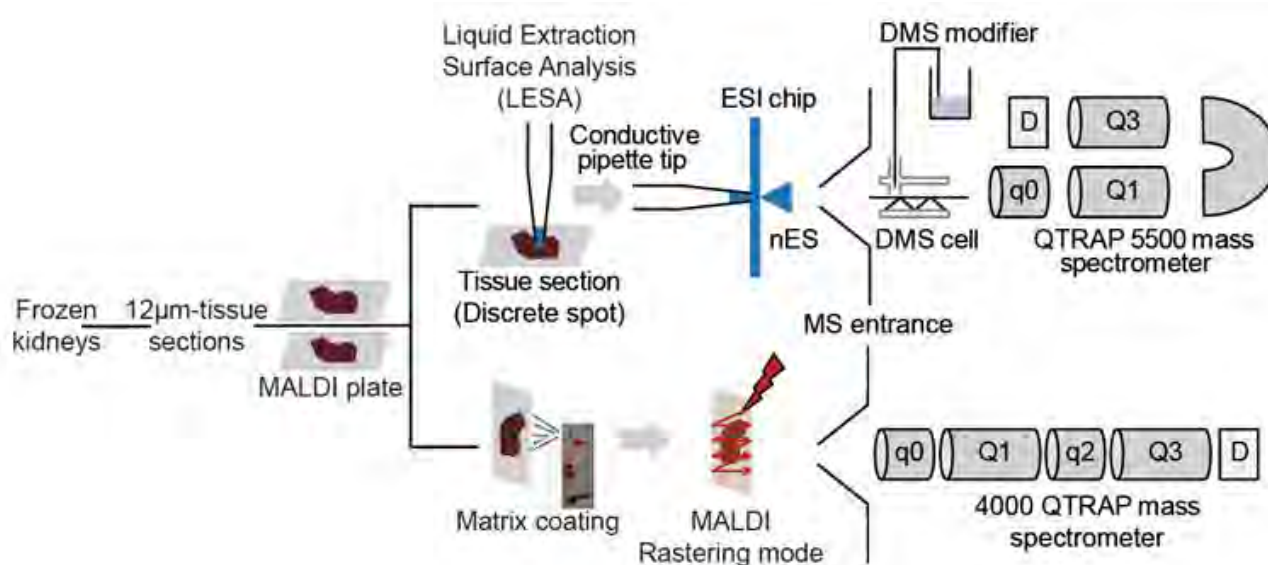


Figure III- 35. Schematic representation of the analytical workflow for direct surface analysis of tissue sections by LESA-DMS (upper part) and MALDI-MS imaging (lower part).

3.5.3.2. Screening and relative quantification of drugs of abuse and their metabolites in human kidney tissues by MALDI-MSI

a) Choice of the MALDI matrix for screening opiates and benzodiazepines

A rapid evaluation of the best MALDI matrix to be used for the analysis of drugs of abuse has been done by manually spotting either CHCA or Cl-CCA on two adjacent tissue sections of the sample B (i.e. positive IA) (Figure III- 36). It became apparent that CHCA gives the best results for morphine-glucuronides and also for diazepam in positive ionization mode. CHCA is thus chosen for further analysis.

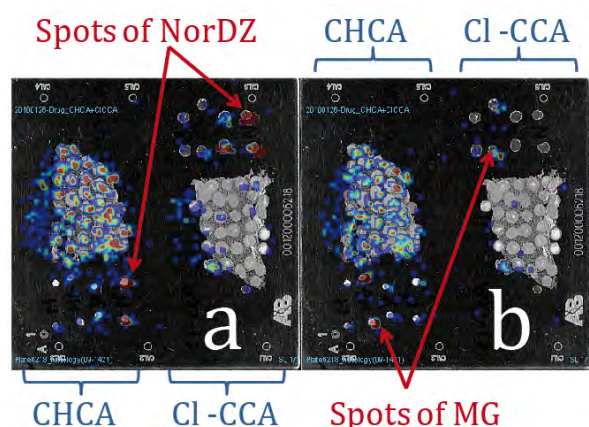


Figure III- 36. Overlay of optical images and MALDI-MSI images for (a) nordiazepam and (b) morphine glucuronide in sample B. 200 nL-droplets of CHCA and Cl-CCA were spotted on two adjacent tissue sections.

b) Determination of the best solvent composition to dissolve the MALDI matrix

The choice of the solvent used for the preparation of the matrix is of utmost importance, as it can promote the extraction of the analytes from the tissue section, and thus affect the sensitivity of the method. Solvent mixtures containing high organic content provide higher extraction efficiency of low molecular weight compounds, and offer the advantage of relatively fast drying. This prevents the potential delocalization of the analytes within the sample. Two acidic solvent mixtures were compared, based on 60% methanol (MeOH) or acetonitrile (ACN) and 0.1%TFA. Four tissue sections from sample A and three sections from sample B were analyzed and the averaged signals obtained are presented Figure III- 37. The best sensitivity, determined by comparison between sample B and sample A (negative sample used as control), is given by using methanol in the solvent mixture used to dissolve CHCA matrix.

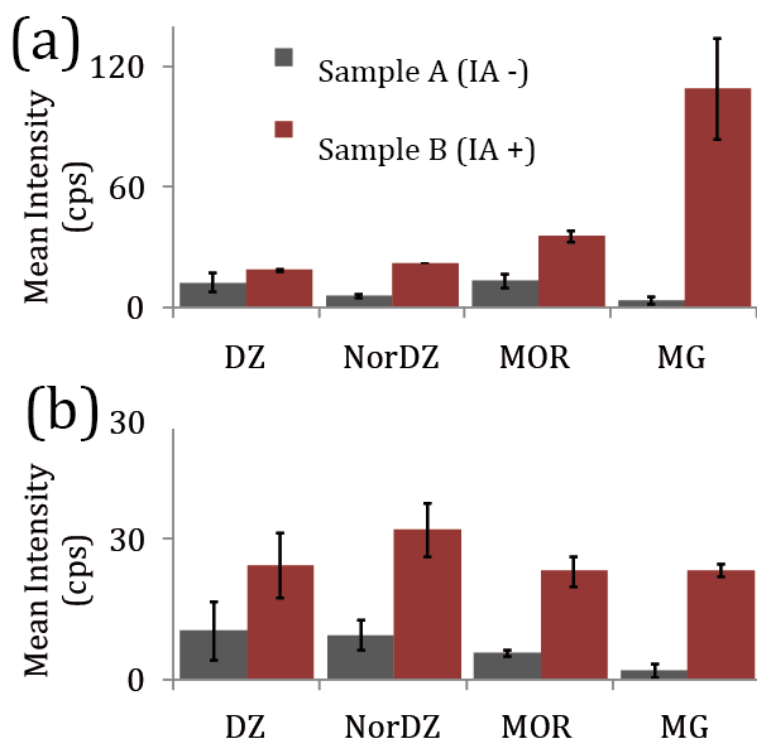


Figure III- 37. Effect of the solvent used for the dissolution of the matrices on the sensitivity of the analytes. (a) CHCA dissolved in 60%MeOH + 0.1% TFA; (b) CHCA dissolved in 60%ACN + 0.1%TFA.

c) Investigation of morphine and diazepam and their respective metabolites in postmortem human tissues

The simultaneous screening of multiple compounds and their relative quantification is performed in the selected reaction monitoring (SRM) mode. The MS duty cycle (250 ms) set in the SRM mode is compatible with a high sample stage velocity operated in rastering mode (i.e. 2 mm/s), and leads to the analysis of a total MALDI plate (44 x 44 mm) in less than 40 minutes with an image resolution of 500 μm . Note the overall analysis time can be decreased by increasing the distance between two raster lines, which is possible when single organs are analyzed.

MALDI-SRM/MSI enables the detection of both MOR and DZ and their metabolites, namely glucuronide metabolites and nordiazepam, respectively, in sample B (Figure III- 37 and Figure III- 38.a) which correlates previous determination by immunoassays.

The structural confirmation of the four analytes was performed in the MS/MS mode called enhanced product ion (EPI) scan, and the spectra were compared with MS/MS spectra generated by the analysis of standard solutions by electrospray (ESI) – EPI analysis (Figure III- 38.b). The structures of the compounds are confirmed by EPI experiments on regions where the compounds were previously detected (as shown Figure III- 38.b for MG) Intake of morphine can be concluded in this case in agreement with routine analysis performed using GC-MS. It is worth noting that morphine glucuronide can actually be present in two forms: morphine-3-glucuronide and morphine-6-glucuronide (this point will be discussed Chapter 4, Part 3). However, the current approach does not allow the differentiation of the 2 isomeric metabolites.

Regarding the determination of diazepam intake, diazepam could not be detected in sample B, whereas immunoassays gave positive results to benzodiazepines and it could be quantified by GC-ECD. So far, one cannot determine if the method is not sensitive enough to allow its detection, or if complete metabolization of the drug occurred. In this case, however, diazepam intake can be undoubtedly proven by the detection and confirmation of the metabolite NorDZ (Figure III- 38.b).

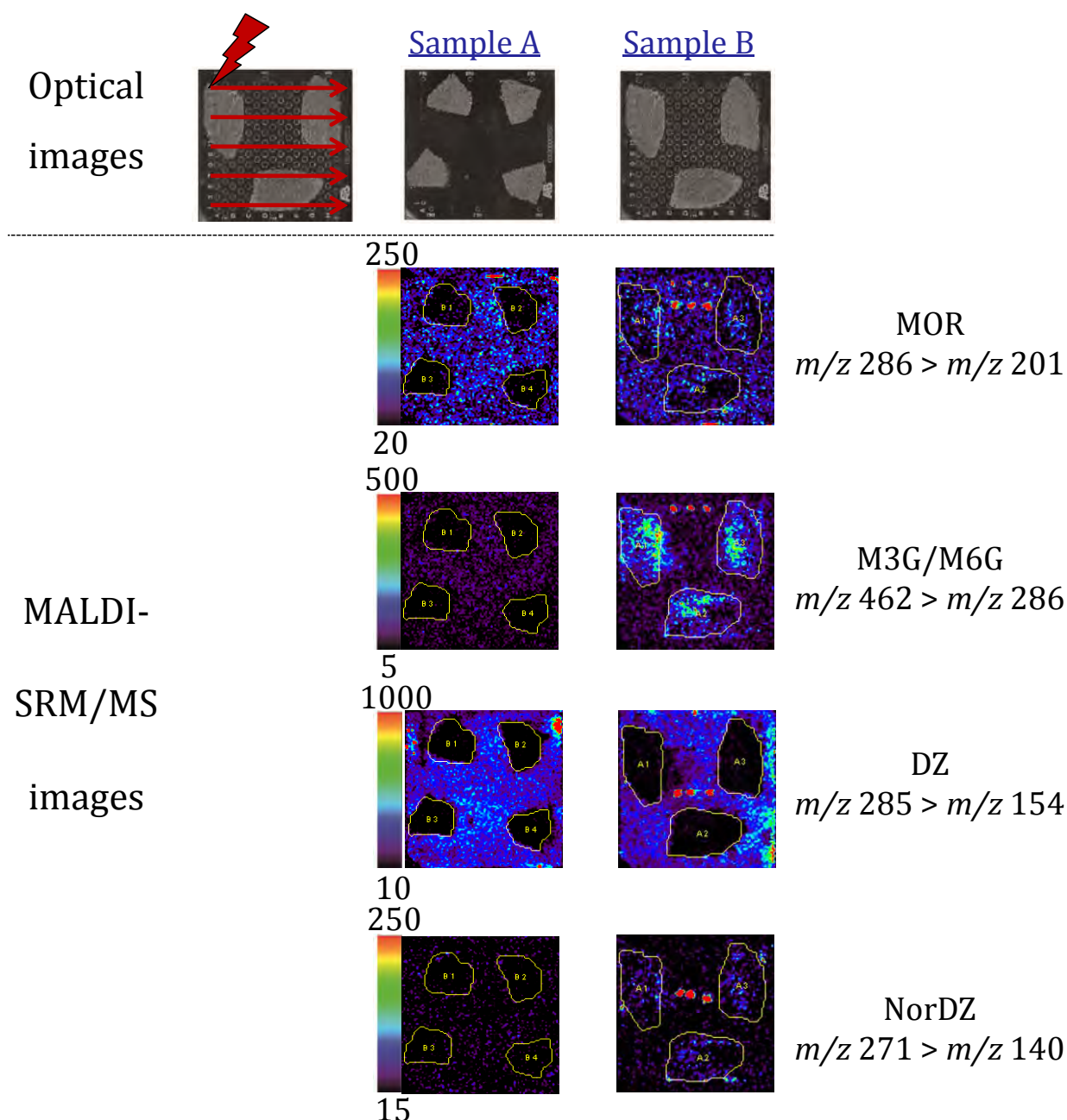


Figure III- 38.a. MALDI-SRM/MS images based on the respective SRM traces for morphine (MOR), morphine-glucuronide metabolites (M3G/M6G), diazepam (DZ) and diazepam metabolite: nodiazepam (norDZ) in postmortem human kidney tissue sections. Sample A is the sample given negative by immunoassays (IA). Sample B is the sample given positive to opiates and benzodiazepines by IA. To generate the MALDI-SRM/MS images: 89 lines horizontal lines were rastered at a plate speed of 2 mm/s; 4 analytes were monitored per run; the total MS scan time was of 250 ms. The pixel size is of 500x500 μm^2 . The total analysis time was 40 min per sample.

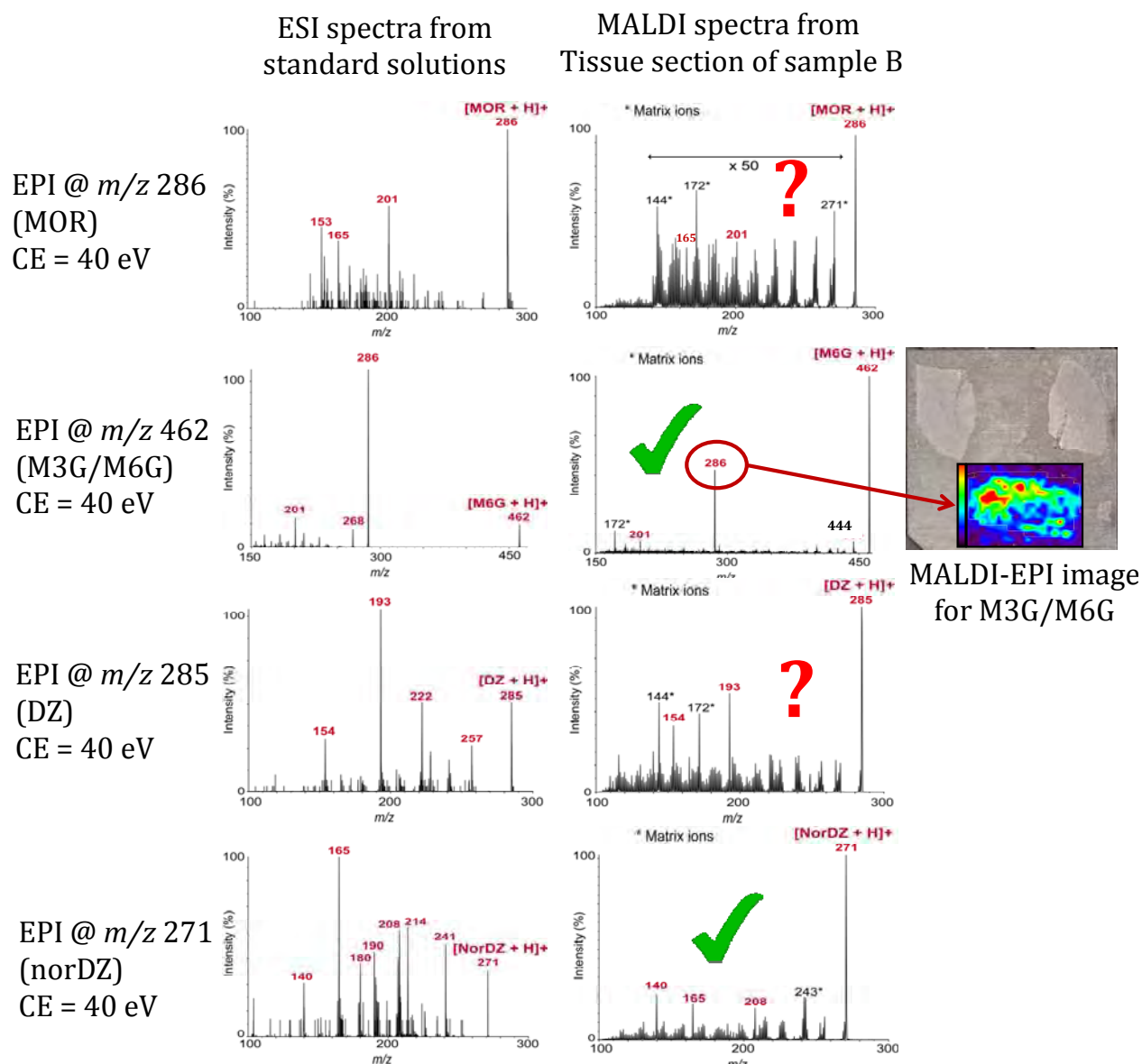


Figure III- 39.b. Structural confirmation for the glucuronide metabolites of morphine M3G and/or M6G. Up: extracted ion image at m/z 286 from MG MALDI-MSI (EPI) experiment (image resolution = 1 mm). Bottom: MS/MS spectrum of $[MG+H]^+$ at m/z 462.

d) MALDI-MS/MS Imaging of cocaine and its metabolites

The approach has also been applied to the screening and relative quantification of cocaine and its two isomers BZE and NCOC in sample C, of which signal recorded within tissue sections by MALDI-SRM/MSI will be compared to the signal recorded from the reference sample A for relative quantification (Figure III- 40.a,b). If MALDI-SRM/MSI allows the detection of cocaine, however, it does not allow the separation of isobaric compounds, such as BZE and NCOC which both contribute to the signal given by the SRM transition m/z 290 > m/z 168 (Figure III- 40.c). A solution to this problem is to monitor a second SRM transition specific for NCOC (i.e. m/z 290 > m/z 136), but this is at the expense of sensitivity (Figure III- 40.b). Thus, only BZE can be detected.

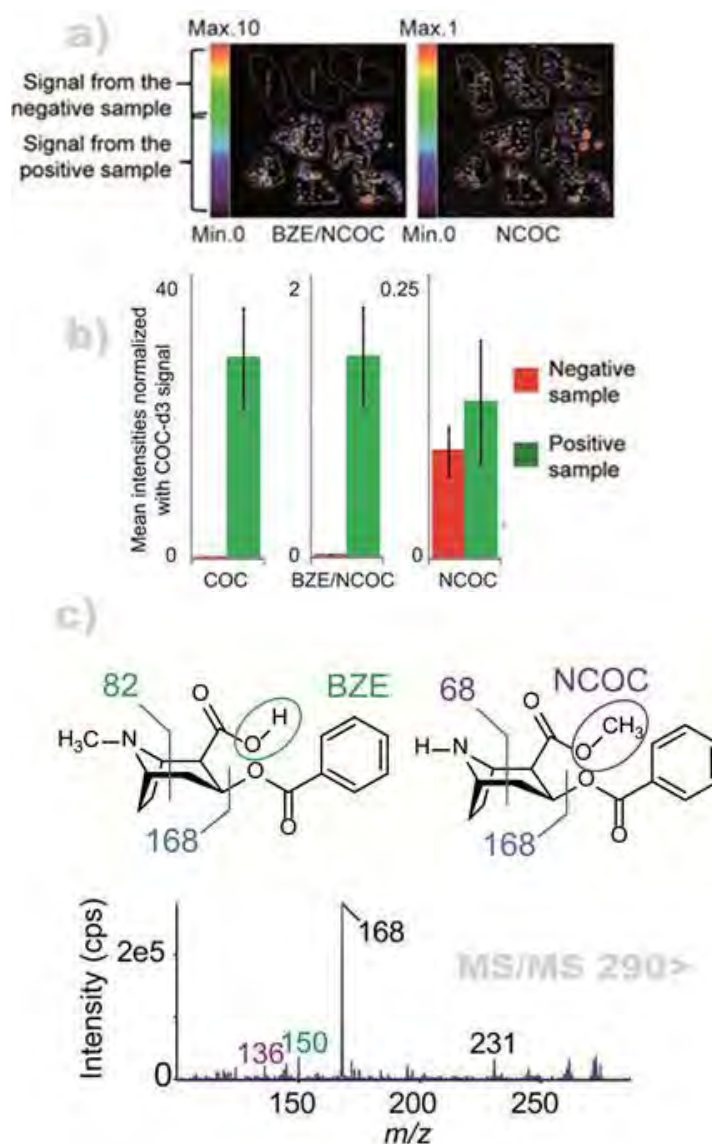


Figure III- 40. Screening and relative quantification of cocaine and its two isomeric metabolites by MALDI-MS/MS imaging. a) MALDI-SRM/MS images based on the SRM response of targeted compounds from kidney samples; b) relative quantification based on the mean signals detected in tissue sections; c) structures of BZE and NCOC and identification of main ions and EPI spectrum at m/z 290 (CE = 30 eV) in sample C. Negative sample refers to sample A. Positive sample refers to sample C.

3.5.3.3. Surface sampling and differential ion mobility spectrometry for the direct analysis of illicit drug metabolites isomers from tissue sections

To overcome this issue of isomeric metabolites encountered with the MALDI-MSI approach, a new analytical strategy is proposed herein, based on the combination of liquid extraction surface analysis (LESA) and differential ion mobility spectrometry mass spectrometry (DMS-MS). DMS is a gas phase separation of ions at atmospheric pressure, based on the difference in ionic mobility at low and high electrical field.¹³⁴ It has been demonstrated that the addition of an organic modifier in the gas phase considerably enhances the separation by the dynamic formation of clusters between the modifier and the ions.¹³⁵⁻¹³⁷

The following section gives an overview of the potential of the platform, which will be detailed in Chapter 4, Part 3.

a) Evaluation of different DMS modifiers for the separation of cocaine metabolites isomers

The evaluation of different organic modifiers (ACN, MeOH, EtOH, toluene and acetone) to enhance the gas-phase separation of BZE and NCOC isomers is made by infusing a solution containing 0.1 µg/mL of cocaine and its metabolites. The addition of acetone, ACN, toluene in the transporting gas as DMS modifiers enables the separation of isomeric metabolites BZE and NCOC (Figure III- 40).

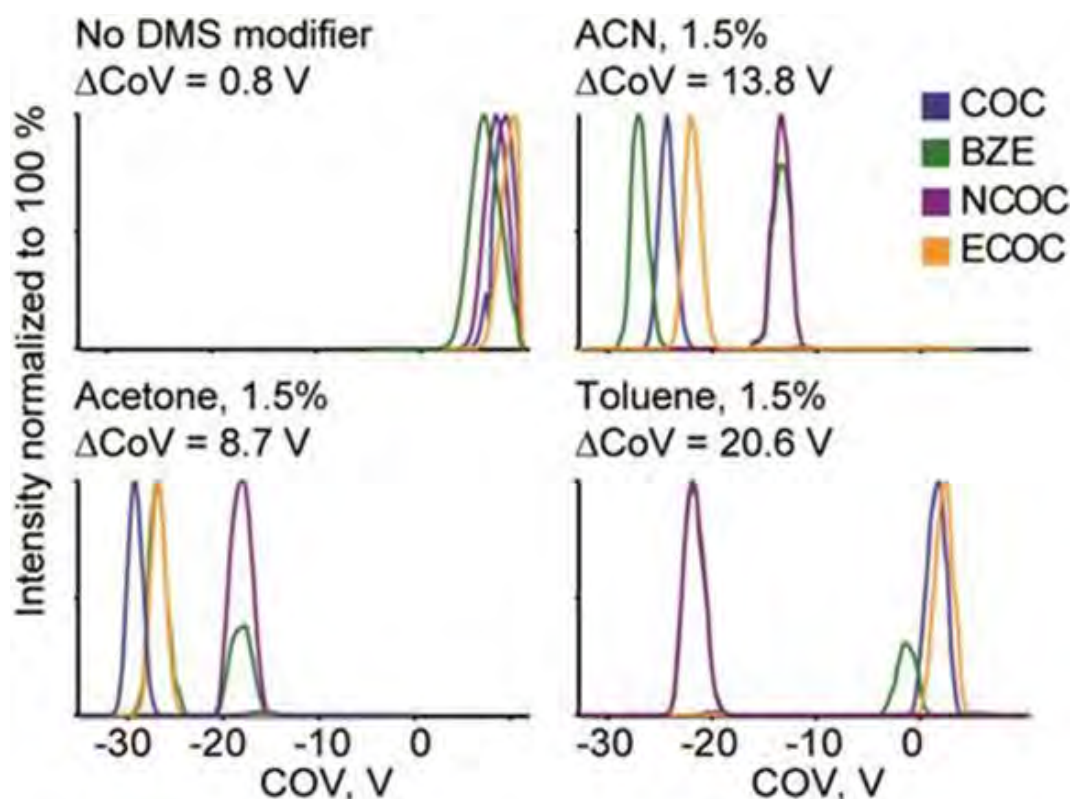


Figure III- 41. Evaluation of different DMS modifiers for the separation of BZE and NCOC. The SRM transitions monitored were: COC: m/z 304 > m/z 182; BZE/NCOC: m/z 290 > m/z 168; NCOC: m/z 290 > m/z 136 and ethylcocaine (ECOC): m/z 318 > m/z 196. The signal is recorded by ramping the compensation voltage.

b) Investigation of cocaine and its isomeric metabolites benzoylecgonine and norcocaine in tissue sections by LESA-DMS-MS

To complement the fast screening of drugs of abuse achieved with MALDI-MSI, a strategy combining liquid extraction surface analysis (LESA) with differential ion mobility spectrometry (DMS) is proposed. The results shown in Figure III- 42 confirm the presence of both BZE and NCOC in the positive sample. The structural identification in both MS/MS and MS³ spectra recorded at specific compensation voltage (see Chapter 4) allow confirmation of the structure of both analytes with a high level of selectivity, as the specific ions detected for each one are not overlapping.

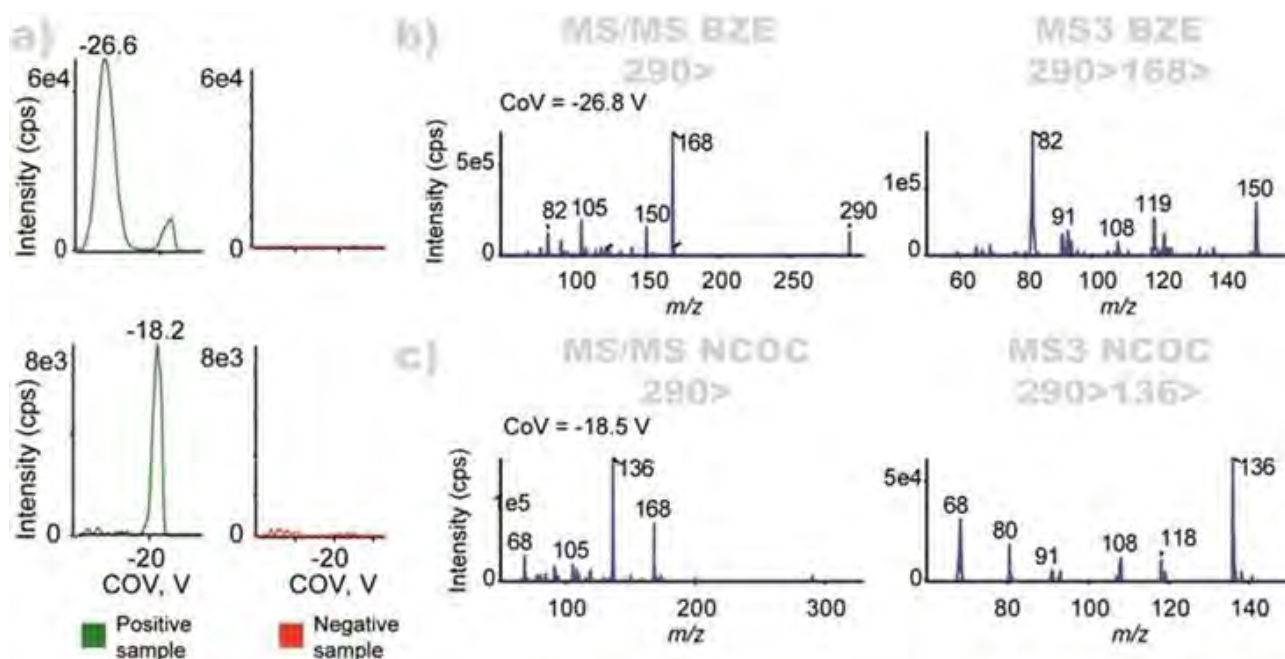


Figure III- 42. LESA-DMS-MS analysis of tissue sections. a) SRM traces from LESA extracts from kidneys for BZE (CoV = -26.6 V) and NCOC (CoV = -18.2 V); structural confirmation made from liquid extracts from positive sample for b) BZE and c) NCOC. ACN1.5 % was used as DMS modifier. The analytes can be identified by the following specific ions: m/z 82 and m/z 150 (loss of H_2O from the fragment m/z 168) for BZE; m/z 68 and m/z 136 (loss of $O=CH_2$ from m/z 168) for NCOC.

3.5.6. Conclusion and perspectives

The present work demonstrates the capability of MALDI-MSI for the rapid screening of drugs of abuse and their metabolites in postmortem human tissue for forensics investigation.

One of the strengths of the approach is the straightforward sample preparation preceding the MS analysis. Contrary to other techniques such as LC-MS or GC-MS that require organ dissection and homogenization, the sample preparation is easy and rapidly achieved, from organ ablation to matrix coating, as long as the matrix is sprayed homogeneously over the whole surface of the sample.

Performed on a MALDI-QqQ_{LIT} platform, the MSI experiments benefit from the fast duty cycle of the selected reaction monitoring mode, which allows a rapid screening of targeted compounds. In the experimental setup designed to prove the suitability of the approach, a single MALDI plate (of which dimensions are 44 x 44 mm), where several samples can be mounted, was analyzed in 40 minutes. However, it is worth noting that experimental parameters, such as the sample stage velocity and the distance between 2 raster lines, can be modified to reduce the analysis time, as long as enough data are acquired per sample to obtain suitable statistics. The influence of such parameters on the analyte response will be discussed in Chapter 5, Part 2.

In addition, the SRM mode also allows the relative quantification of drugs and their metabolites, achieved by comparison with a reference sample, previously analyzed by immunoassays and GC-MS to confirm the non-detection of the analytes in this sample. Finally, the mass analyzer operated in trap mode permits confirmatory analysis for ultimate selectivity and sensitivity, and increases the quality of the data to prove the potential consumption of illicit substances for forensics purpose.

These experiments show that the main challenge for the MALDI-SRM/MSI approach is the analysis of isobaric compounds, as shown for cocaine and two of its isomeric metabolites, benzoylecgonine and norcocaine. To overcome this issue, the study introduces a new complementary analytical platform based on a liquid extraction of analytes from tissue sections, combined with differential ion mobility spectrometry. The results given by both MALDI-MSI and LESA-DMS-MS are in accordance, and enable the detection and confirmation of cocaine metabolites in tissue samples with a high level of selectivity provided by the ion mobility dimension after the surface sampling.

The hyphenation of LESA-DMS with a triple quadrupole linear ion trap mass analyzer increases the selectivity of the approach. This novel platform provides new perspectives for the direct analysis of drugs and metabolite isomers from complex biological surfaces, since DMS can act as a chromatographic separation, and thus increases the selectivity compared to MALDI-MSI directly performed without chromatographic separation.

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Appendix: Research article in preparation

“Identification of the first BAHD involved in tropane alkaloid biosynthesis”

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Abstract

The esterification of 2-carbomethoxy-3 β -tropine with benzoic acid is the final step in the biosynthetic pathway leading to the production of cocaine in *Erythroxylum coca*. Here we report the identification and characterization of cocaine synthase, a member of the BAHD family of plant acyltransferases. Cocaine synthase is capable of producing both cocaine and cinnamoylcocaine via the activated benzoyl- or cinnamoyl-CoA thioesters, respectively. Cocaine synthase activity is highest in the young developing leaf tissues and accumulates in the palisade parenchyma and spongy mesophyll. This data correlates with the homogenous distribution pattern of cocaine and cinnamoylcocaine as visualized using MALDI-MSI. Our findings provide further evidence that tropane alkaloid biosynthesis in Erythroxylaceae occurs in the above ground portions of the plant and contrasts with what is known about tropane alkaloid biosynthesis in Solanaceae.

Keywords

Cocaine, Cinnamoylcocaine, Tropane alkaloids, MALDI-MSI, *Erythroxylum coca*, BAHD acyltransferase

Introduction

Erythroxylum coca has been cultivated for religious and medicinal purposes for more than 8000 years ¹. It is believed that this is one of the first plants to be exclusively cultivated based solely upon its pharmacological properties. The result of long term cultivation and selection for increasing alkaloid content has led to the existence of several cultivars containing cocaine in amounts of up to 1% dry weight in the plant leaves ². Cocaine (**2**), the benzoate ester of 2-carbomethoxy-3 β -tropine (methylecgonine) (**10**), belongs to a class of alkaloids commonly referred to as tropane alkaloids. Tropane alkaloids are defined by a common chemical substructure, the azabicyclo[3.2.1]octane skeleton (**1**) or tropane nucleus (**Fig. 1**). Esterifications and hydroxylations of the tropane skeleton are common in nature, and more than 200 tropane alkaloids with drastically different pharmacological activities are known to exist ³. The physiological interactions mediated by these compounds have been largely attributed to the modification and decorations made to the tropane skeleton. The methylated nitrogen atom in the tropane skeleton mimics acetylcholine and thereby inhibits the muscarinic acetylcholine receptors ⁴. Furthermore, the stereochemical orientations at both the C-2 and C-3 positions are important for binding at the dopamine receptor ^{5,6}, with the strongest affinity being found on compounds containing an aromatic ring connected directly or indirectly to the 3 β position ⁷. This in turn explains why cocaine is used for both its anesthetic and euphorigenic properties. Extensive research on tropane alkaloid biosynthesis using isotopically labeled compounds and chemical analyses has been carried out using *E. coca* in the past, establishing a theoretical biosynthetic pathway to cocaine (**Supplementary Fig. 1**) ⁸. In short, the early biosynthetic steps go through ornithine or arginine to produce the committed polyamine known as *N*-methylputrescine (**5**). After oxidation of *N*-methylputrescine to 4-methylaminobutanal (**6**), which undergoes spontaneous cyclization to an *N*-methyl- Δ^1 -pyrrolinium cation (**7**), two acetyl units are added. The oxobutanoic acid intermediate (**8**) then cyclizes to form the tropane intermediate called methylecgonone (**9**). In the penultimate biosynthetic step to cocaine,

methylecgonone is reduced to form methylecgonine (**10**). This reaction is catalyzed by the enzyme methylecgonone reductase (MecgoR) ⁹, which was the first structural gene of cocaine biosynthesis to be identified.

The last step in cocaine biosynthesis, esterification of methylecgonine, was hypothesized to utilize benzoyl coenzyme A as the activated acyl donor ¹⁰. Shortly thereafter, the origin of this benzoyl moiety in cocaine was determined to be derived from cinnamic acid. However, this study could not determine whether the benzoic acid comes from benzoyl-CoA or via benzaldehyde ¹¹. In parallel, enzyme activities responsible for the acetylation of tropane alkaloids were purified, but no structural genes were ever isolated ^{12,13}. In plants, acylation reactions of secondary metabolites are performed by several acyltransferase families, namely the THT/SHT, BAHD and SCPL acyltransferases. However, of these three groups only the BAHD acyltransferases are known to utilize activated acyl coenzyme A thioesters ¹⁴. In this study, we report the identification and characterization of the enzymes responsible for the last biosynthetic step in the formation of cocaine in *E. coca*. This particular step is of high biological importance since it converts a molecule with little physiological activity, methylecgonine, into the medically active end product of the pathway, cocaine ¹⁵.

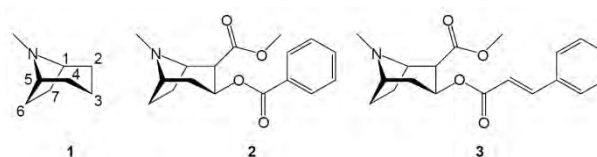


Figure 1 | Structures of selected tropane alkaloids. (1) numbered tropane nucleus, (2) cocaine and (3) cinnamoylcocaine.

Results

Exploration of cocaine synthase activity

To investigate whether cinnamoylcocaine and cocaine formation is dependent upon coenzyme A thioesters, enzyme assays were performed on crude plant *E. coca* extracts using methylecgonine and either cinnamoyl- or benzoyl-CoA. Ester forming activities were found in all tissues extracts with the exception of the roots (**Fig. 2a**). The highest enzyme activities are present in the

early leaf stages (L1 and L2) with 313 ± 20 pkat / mg (fresh weight) for benzoyl-CoA and 1285 ± 97 pkat / mg (fresh weight) for cinnamoyl-CoA as substrate. Stem, flower and leaf stage 3 (L3) extracts were reduced by 20%, 40% and 60%, respectively when compared to leaf stages L1 and L2. Overall metabolite levels correlate with enzyme activities in all the tissues tested (**Fig. 2b**). Cocaine, cinnamoylcocaine and methylecgonine are highest in the early leaf stages (L1 and L2) followed by leaf stage 3 stem and flower. None of these three metabolites were detected in the roots.

Cocaine synthase is a BAHD

Recently, six BAHD acyl transferases were reported from an *E. coca* λ ZAPII cDNA library¹⁶. Additionally the screening of a transcriptome database made from *E. coca* early leaf tissues (L1 and L2) yielded two more BAHD sequences designated EcBAHD7 and EcBAHD8. All eight EcBAHD's were heterologously expressed in *Escherichia coli* and the resulting recombinant proteins were purified using Ni-chelate chromatography. Verification of protein expression for all eight EcBAHDs was achieved using immunoblot analysis with anti-His antibodies (**Supplementary Fig. 2a**). The recombinant purified proteins were tested in enzyme assays using the substrates methylecgonine and benzoyl-CoA. Of the eight individual proteins tested only EcBAHD7 and EcBAHD8 exhibited ester forming activity. A sequence alignment of EcBAHD7 and BAHD8 reveals that both enzymes share 77.3% identity positions at the amino acid level. Both EcBAHD7 and EcBAHD8 contain recognizable BAHD motifs including the DFGWG motif found near the C-terminus as well as the HxxxD motif which is critical for a catalytic function¹⁴. Phylogenetic analysis reveals that EcBAHD7 and EcBAHD8 belong to the clade III of the BAHD superfamily (**Fig. 3**). Within this clade EcBAHD7 and EcBAHD8 cluster with three other BAHDs involved in alkaloid biosynthesis. Two of these enzymes, DAT and MAT from Madagascar periwinkle (*Catharanthus roseus*) are involved in the formation of vindoline and minovincine, respectively, both monoterpenoid indole alkaloids, whereas Pun1 has shown to be involved in capsaicin formation in pepper¹⁷.

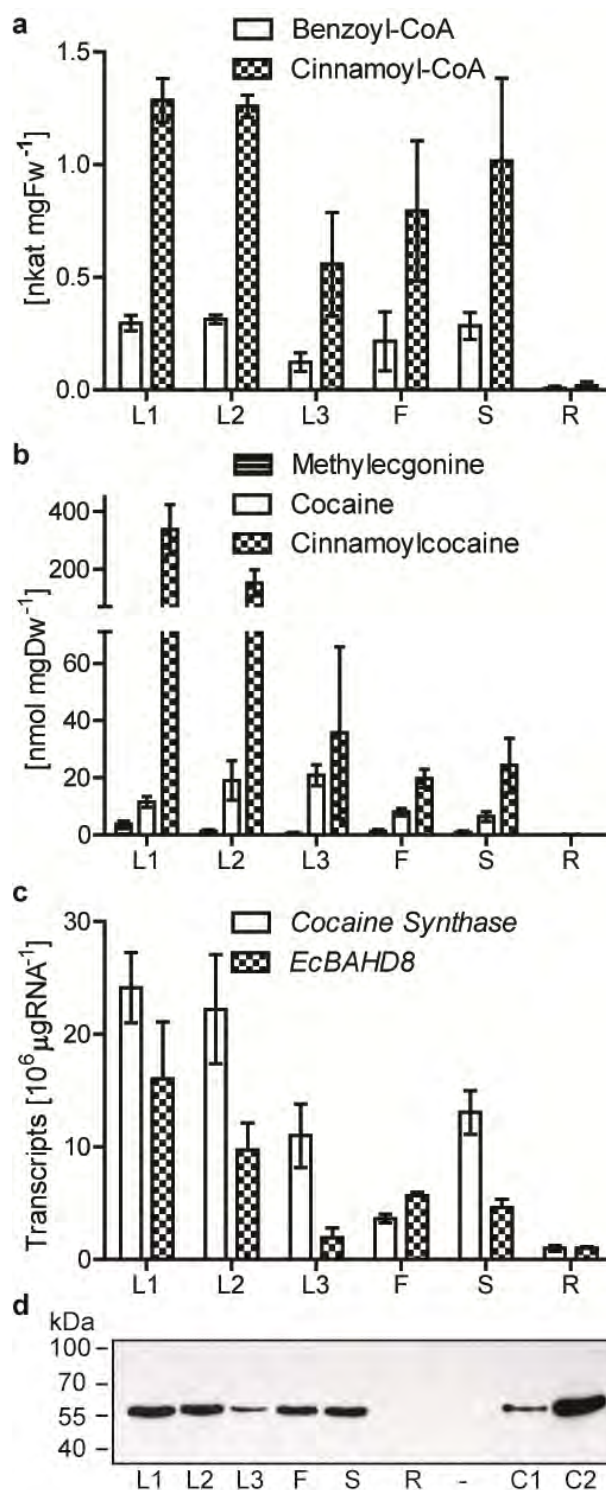


Figure 2 | Enzymatic activity and expression of cocaine synthase and EcBAHD8 compared to metabolite levels of compounds involved in the cocaine pathway, in different *E. coca* organs and development stages. (a) Cocaine and cinnamoylcocaine forming enzyme activity in desalted *E. coca* protein preparations. Enzymatic assays were performed using methylecgonine with either

benzoyl- or cinnamoyl-CoA, and detecting cocaine or cinnamoylcocaine as the reaction products, respectively. Values displayed are means $\pm$ SD of three technical replicates from each of three biological replicates. (b) Quantification of methylecgonine, cocaine and cinnamoylcocaine in different organs and development stages of *E. coca*. Values displayed are means $\pm$ SD of at least three biological replicates. (c) Absolute quantification of RNA transcripts of cocaine synthase and EcBAHD8 in *E. coca*. Values displayed are means $\pm$ SD of three technical replicates from each of three biological replicates. (d) Protein levels were determined by immunoblotting. Samples consisting of 15 μ g of protein extracted from each organ as well as 75 ng (C1) and 150 ng (C2) recombinant Strep-tagged cocaine synthase (50.7 kDa) were run on SDS/PAGE and gels were blotted onto filters. The filters were first probed with anti-cocaine synthase antibodies, followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Polyclonal antibodies recognize cocaine synthase as well as EcBAHD8 (Supplementary Fig. 2b). Bands were visualized by chemiluminescence. Leaf stage 1 (L1), Leaf stage 2 (L2), Leaf stage 3 (L3), Flower (F), Stem (S), Root (R).

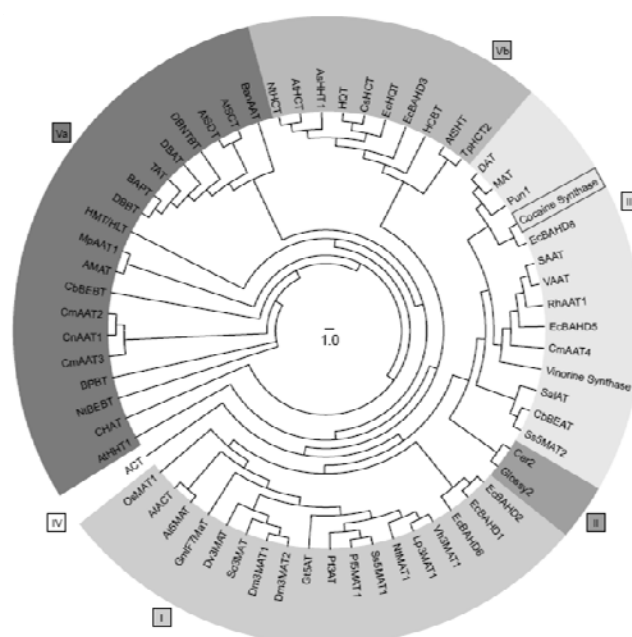


Figure 3 | Phylogenetic relationships of BAHD acyltransferases of plant secondary metabolism. Selected BAHD acyltransferases were aligned using the CLUSTAL X program with standard settings for protein alignment. The phylogenetic tree was built by the Bayesian method using the MRBAYES program. The different clades of BAHD acyltransferases are depicted according to ¹⁴. The scale bar represents 1.0 amino acid substitutions per site. Please refer to Supplementary Table 1 for an explanation of the abbreviated names. Accession number: cocaine synthase (KC140149) and EcBAHD8 (KC140150).

Enzymatic characterization reveals BAHD7 as true cocaine synthase (CS)

Heterologously expression for EcBAHD7 and EcBAHD8 for biochemical characterization was carried out by *Saccharomyces cerevisiae* as host organism. Although abundant protein could be attained from expression in *E. coli*, overall enzyme activities were determined to be optimal in yeast. In addition a codon optimized version of EcBAHD7 was developed to further enhance protein expression levels for biochemical characterization (**Supplementary Fig. 3**). The native EcBAHD7 protein with the addition of StrepTagII (10 amino acids added) was analyzed via gel sizing chromatography and yielded a single peak corresponding to a size of 43.9 kDa.

This data suggests that EcBAHD7, like other characterized acyl transferases, is monomeric¹⁴. The pH optimum of the heterologously expressed EcBAHD7 protein was determined to be 9.4 when catalyzing the esterification of methylecgonine with benzoyl-CoA as the acyl donor. The enzyme activities were reduced to 39% and 44% of maximal activity when using a pH of 9.1 or 10.4, respectively. Similar pH optima were also obtained for the heterologously expressed EcBAHD8.

Temperature stability experiments revealed that the enzymatic activity of EcBAHD7 when incubated for 30 min at 25 °C and 37 °C were reduced by 45 % and 29% respectively when compared to 0 °C control. Higher incubation temperatures led to complete loss of ester forming activity. In contrast, EcBAHD8 did not exhibit any reduction in enzymatic activity when incubated for 30 min at 20 °C and activity levels remained at 96% after incubation at 37 °C. However, higher incubation temperatures also led to complete loss of ester forming activity. Neither, EcBAHD7 or EcBAHD8 were capable of being stimulated by the inclusion of monovalent or divalent cations. A marginal reduction in the ester forming capabilities of both enzymes was observed by performing enzyme assays with the inclusion of 5 mM Na⁺ and K⁺. Complete inhibition of enzyme activity was achieved for EcBAHD7 with Cu²⁺ and Zn²⁺. Strong inhibition (less than 10% maximal activity) was also observed with the addition of Mg²⁺, Co²⁺, Fe²⁺ and EDTA when compared to no added metal cation control. A similar pattern of inhibition was observed for EcBAHD8.

Kinetic values were obtained for EcBAHD7 and EcBAHD8 using methylecgonine, benzoyl-CoA and cinnamoyl-CoA as substrates (**Table 1**). The EcBAHD7 Michaelis-Menten constant (K_m) for methylecgonine and benzoyl-CoA is $369 \pm 26 \mu\text{M}$ and $93 \pm 7 \mu\text{M}$, respectively. When using methylecgonine and cinnamoyl-CoA as substrates, K_m values of $62 \pm 11 \mu\text{M}$ for methylecgonine and $103 \pm 8 \mu\text{M}$ for cinnamoyl-CoA were obtained. K_m values of $843 \pm 64 \mu\text{M}$ and $7 \pm 1 \mu\text{M}$ were calculated for EcBAHD8 when given the substrates methylecgonine and benzoyl-CoA, respectively. Enzyme assays using methylecgonine and cinnamoyl-CoA with EcBAHD8 yielded K_m values of $57 \pm 6 \mu\text{M}$ and 23

$\pm 2 \mu\text{M}$, respectively. Furthermore, substrate inhibition was observed for the CoA thioesters in EcBAHD8 kinetic assays. K_i values of $5 \pm 1 \text{ mM}$ and $14 \pm 3 \text{ mM}$ were observed for benzoyl- and cinnamoyl-CoA, respectively. Comparison of catalytic efficiencies (k_{cat} / K_m) revealed that EcBAHD7 is more than 1,000 fold higher when using methylecgonine and benzoyl-CoA as substrates. The difference increases to 5,000 fold when comparing the catalytic efficiencies of these two enzymes using methylecgonine and cinnamoyl-CoA as substrates. Because EcBAHD8 is far less efficient at catalyzing the production of cocaine or cinnamoylcocaine when compared to the activity of EcBAHD7, we designated EcBAHD7 as a true cocaine synthase (CS).

Relative Activity assays performed using cocaine synthase yielded ester forming activities only when using pseudotropine or methylecgonine as the alcohol containing substrate. No esterified compounds were detected when using tropine or nortropine as substrates. The specific activity of CS for the reaction of methylecgonine and benzoyl-CoA was 4.03 pkat/mg. Reductions of 73%, 81% and 99% were observed when using cinnamoyl-CoA, hexanoyl-CoA or coumaroyl-CoA. Polyclonal antibodies were produced against the heterologously expressed CS protein. The specificity of the polyclonal antibodies as well as the identity of the cocaine forming activity *in planta* was evaluated by immunoprecipitation. The cocaine forming activity in *E. coca* leaf stage 2 (L2) protein extracts was measured at 3.41 pkat / mg and was subsequently reduced to 1.23 pkat / mg. Pre-immune serum did not reduce enzyme activity. Immunoprecipitated proteins were separated on a protein gel and subsequent protein sequencing analysis identified EcCS within the sample. No EcCS was detected in the precipitate formed by the pre-immune serum. Furthermore, we were unable to detect EcBAHD8 in any of these samples.

Table 1 | Kinetic parameters of EcCS and EcBAHD8

		K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]
EcCS	methylecgonine	369 ± 26	10 ± 0	26340
	benzoyl-CoA	93 ± 7	8 ± 0	81192
	methylecgonine	62 ± 11	19 ± 1	301485
EcBAHD8	cinnamoyl-CoA	103 ± 8	46 ± 0	446522
	methylecgonine	843 ± 64	0.02 ± 0.00	24
	benzoyl-CoA	7 ± 1	0.02 ± 0.00	2447
	methylecgonine	57 ± 6	0.003 ± 0.000	54
	cinnamoyl-CoA	93 ± 7	0.005 ± 0.000	23

Cocaine synthase and BAHD8 are localized to above ground tissue

Both *cocaine synthase* (CS) and *EcBAHD8* gene expression is highest in the early leaf stages (L1 and L2) (**Fig. 2c**). Overall, cocaine synthase transcript levels were at least a 2 fold higher in all the tissues tested with the exception of the flowers. In this case, there was no difference in transcript levels. Transcript abundance for CS and *EcBAHD8* were reduced by 50% in the mature leaves (L3) and stems when compared to the early leaf stages. While transcripts could be detected for both genes in the roots they, exhibited more than a 25 fold reduction when compared to L2 stage leaves.

Protein levels in different plant organs were evaluated for cocaine synthase using immunoblot analysis. The results revealed that CS protein is detected in all green tissues including the leaves, flowers and stems. However, no cocaine synthase protein was detected in the roots (**Fig. 2d**).

Cocaine synthase is localized to the palisade parenchyma

Based on the immunoblot results obtained from various organs, green tissues including flowers and stems were used for subsequent tissue level immunolocalization experiments. In addition, anti-cocaine antibodies were used to localize cocaine in the same tissues. **Fig. 4** shows cross sections for stage 1 leaves and stems as well as longitudinal sections made from the flowers. Preimmune serum was employed as negative control and the fluorescence signal obtained from these samples is considered unspecific. All panels in **Fig. 4** are overlays of fluorophore and autofluorescence channels. Strong fluorescence signal was observed following the use of anti-cocaine synthase and anti-cocaine antibodies. Cocaine is localized between the upper and lower epidermis with fluorescence signal detection

strongest in the palisade and spongy mesophyll. A similar pattern can be observed with the CS protein. The strongest signals appear in the palisade with a diffused distribution observable in the spongy mesophyll. In the flower, CS protein accumulates in the tapetum cells of the anther. In addition, the cocaine synthase protein was observed to accumulate in the ground tissue of green stems. The upper or lower epidermal tissues including the cuticle were devoid of fluorescence signal attributed to anti-cocaine antibodies.

Cocaine is evenly distributed within the whole leaf

In order to observe accumulation patterns for methylecgonine, cinnamoylcocaine and cocaine, MALDI imaging techniques were employed. These three compounds were detected by selected reaction monitoring (SRM) acquisition mode and their identities further confirmed by enhanced product ion MS/MS experiments. **Fig. 5** displays homogenous distribution for the three compounds in intact leaf samples of all leaf stages (L1, L2, L3) (**Supplementary Fig. 4**). The cocaine signal was the highest of all three metabolite signals followed by cinnamoylcocaine and methylecgonine. Furthermore, there is a difference in signal intensity between the adaxial and abaxial surfaces of the leaf stages 2 and 3 (L2 and L3). The cocaine signal intensity is highest in the abaxial surface of leaf stage 2 and 3 (L2 and L3) compared to the adaxial leaf surface. In contrast, the signal intensity of cinnamoyl cocaine is higher in the adaxial surface of leaf stage 2 and 3 (L2 and L3). The methylecgonine signal intensity is overall very low, but is higher in the adaxial leaf surface of leaf stage 2 (L2) and the abaxial leaf surface of leaf stage 3 (L3) compared to the opposite leaf surface. An equal signal intensity distribution of all three metabolites can be observed in adaxial and abaxial leaf surfaces of leaf stage 1 (L1).

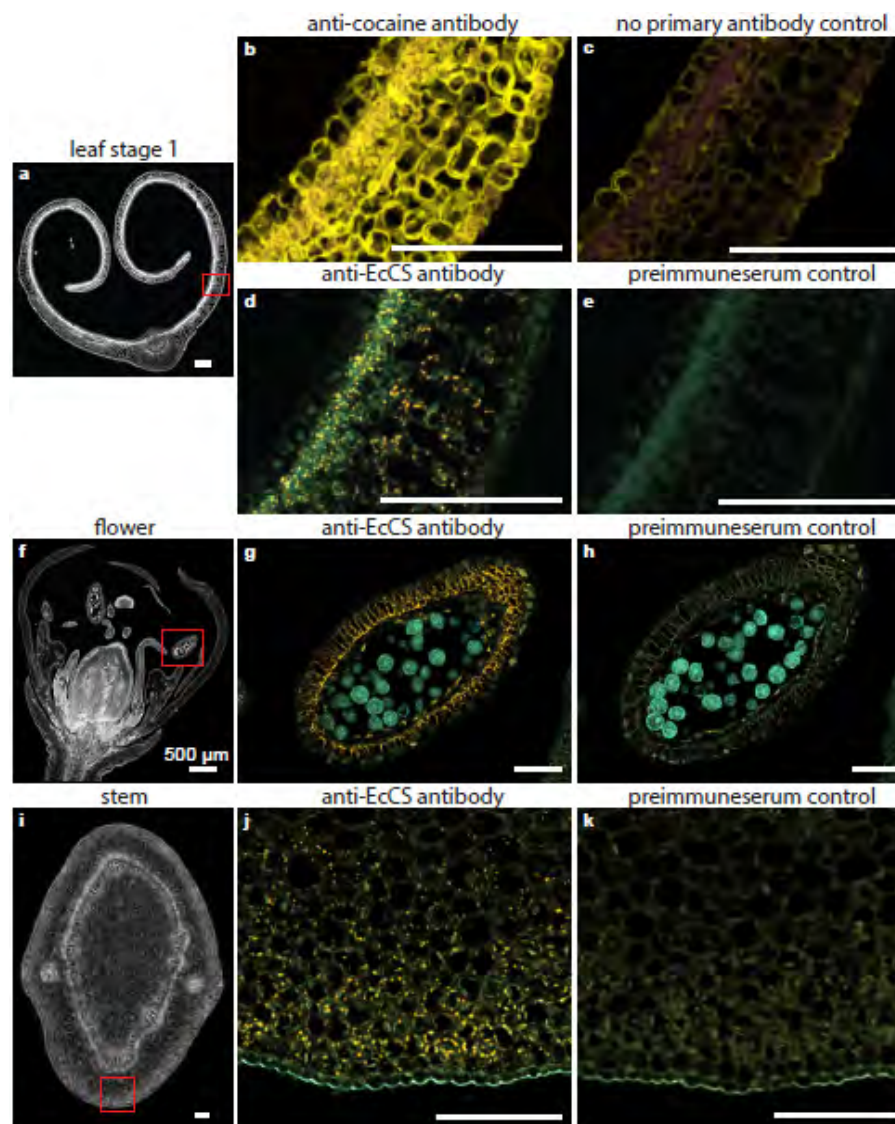


Figure 4 | Immunolocalization of cocaine and cocaine synthase. Fluorescence micrographs of immunolabelled cross-section of different *E. coca* organs. All scale bars represent 100 μm unless otherwise indicated. (a) Overview of leaf stage 1 cross-section with the region of interest marked by a red rectangle. (b) and (c) Leaf stage 1 cross-section immunolabelled with monoclonal anti-cocaine antibodies and no primary antibody, respectively. Fluorescence signal of secondary antibody shown in yellow. Background autofluorescence shown in purple. (d) and (e) Leaf stage 1 cross-section immunolabelled with polyclonal anti-cocaine synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. (f) Overview of flower cross-section with the region of interest marked by a red rectangle. (g) and (h) flower cross-section immunolabelled with polyclonal anti-cocaine synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. (i) Overview of stem cross-section with the region of interest marked by a red rectangle. (j) and (k) Stem cross-section immunolabelled with polyclonal anti-Cocaine Synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. Single sections were probed with primary antibody (anti-cocaine, anti-cocaine synthase or preimmune serum) and secondary antibody (anti-rabbit conjugated to horseradish peroxidase) and subsequently assayed with fluorescent tyramide substrate. Excitation of fluorophore for cocaine imaging was at 543 nm and detection using a BP 585–615 filter. Plant autofluorescence was excited at 488 nm and detected using LP 505nm filter. For imaging of cocaine synthase excitation of fluorophore was at 561 nm and detection using 585–614 nm lambda channels. Plant autofluorescence was excited at 488 nm and detected using 495-534 nm lambda channels. All pictures are overlays of fluorophore and autofluorescence channels.

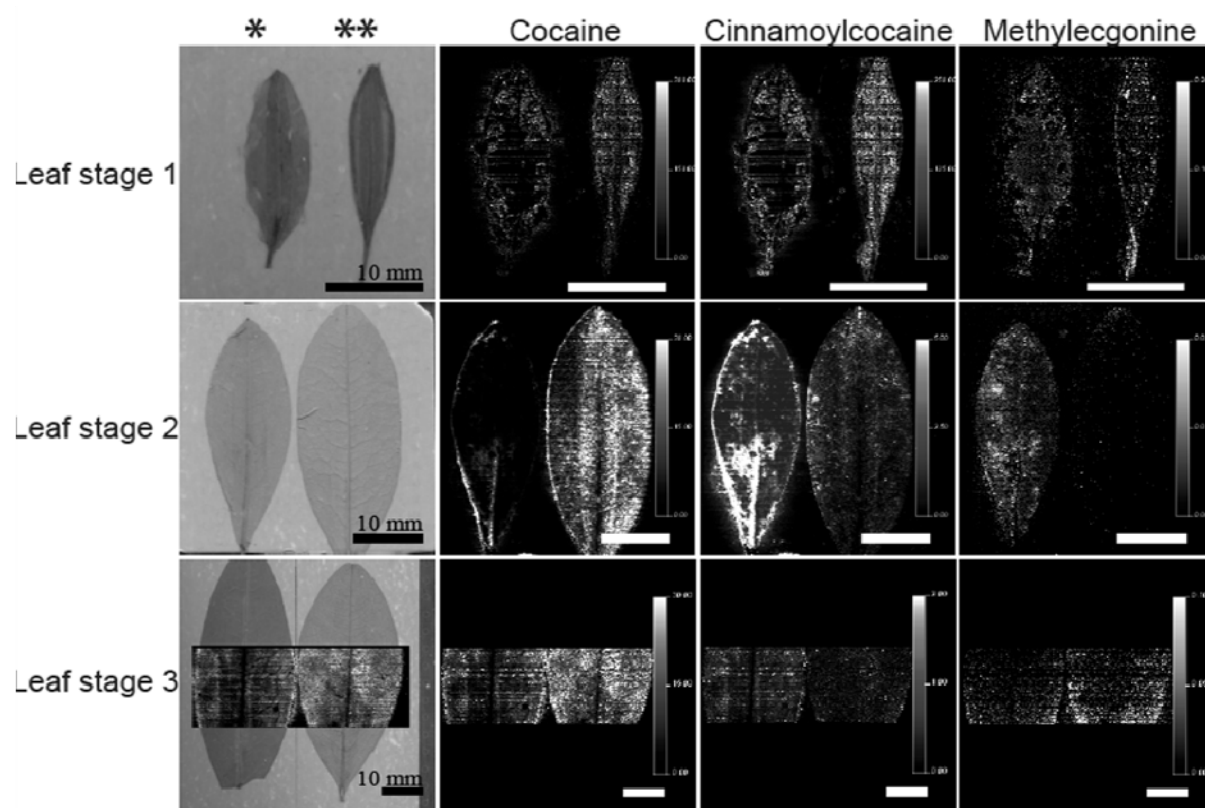


Figure 5 | MALDI-MSI of different developmental stages of *E. coca* leaves. MALDI-SRM/MS images based on the respective SRM traces for targeted analytes. Cocaine trace is shown in blue at m/z 304 > m/z 182, cinnamoylcocaine trace is shown in pink at m/z 330 > m/z 182 and methylecgonine trace is shown in green at m/z 200 > m/z 182 in leaf stage 1, 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine-d₃. The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine-d₃: white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. *adaxial leaf surface; **abaxial leaf surface. MS image resolution: pixels of 50x50 μ m.

Discussion

Tropane alkaloids (TAs) are commonly modified via esterification of the hydroxyl function at the C-3 position of the tropane ring. The four major plant lineages known to contain TA producing plant species all exhibit examples in which this modification occurs (CITE ABR REVIEW JIRSCHITZKA if published by this time). Coenzyme A (CoA) activated thioesters were first suggested to be the actual substrates for the corresponding acylation reaction occurring in *Erythroxylum coca*¹⁰. Shortly thereafter, two CoA dependent acyltransferases involved in TA modification were purified from *Datura stramonium*, a member of the Solanaceae. However, the sequence of the proteins and their corresponding genes were never reported¹³. After confirming, that the cocaine synthase reaction in *E. coca* does indeed use benzoyl-CoA as a cosubstrate, we began to look for the most likely candidates responsible from a 454 transcriptome database obtained from young leaf tissues. Given the CoA dependent nature of the enzyme reaction as well as the physical properties reported for the tigloyl-CoA:pseudotropine acyl transferase from *D. stramonium* we hypothesized that cocaine synthase is most likely a member of the BAHD superfamily¹².

BAHD acyltransferases are well known for participating in the modification of secondary metabolites producing both esters and amides¹⁴. A total of eight BAHD-like acyltransferases were subsequently identified in our databases. From these eight only two exhibited cocaine synthase activity. Another member from this group was recently reported to be EchQT, responsible for making 4-coumaroylquinic acid, a compound that is likely to be involved in the storage of cocaine and cinnamoylcocaine *in planta*¹⁶. The activities of the remaining five BAHD-like enzymes remain to be determined. It is expected that the *E. coca* genome should contain more members of the BAHD acyltransferase family since species like *Arabidopsis thaliana* and *Populus trichocarpa* contain between 64 and 149 unique members, respectively^{14,18}.

Although cocaine synthase and EcBAHD8 share over 77% sequence identity at the amino acid level, cocaine synthase has a much higher efficiency for the production of both cocaine and

cinnamoylcocaine (1,000 fold and 5,000 fold, respectively). The two enzymes share similar Michaelis-Menten constants for methylecgonine and either benzoyl- or cinnamoyl-CoA. Therefore the large catalytic efficiency differences are attributed to very low turnover numbers in respect to EcBAHD8. Moreover, the two purified TA acyltransferases from *D. stramonium* also show similar simple Michaelis-Menten kinetics for their two respective substrates. We observed substrate inhibition kinetics only for EcBAHD8 in respect to the use of either benzoyl- or cinnamoyl-CoA. However the high K_i values of 5 mM and 14 mM, respectively, do not reflect the natural concentration of CoA thioesters reported in plants^{19,20}.

The pH optima for enzyme catalyzed reactions involving the acylation of TA is in the range of 9 to 9.5^{12,21}. This contrasts with pH optima reported for nearly all other BAHD members. In those cases the alcohol containing substrates are nearly always neutral in charge. At pH's above 8 the nitrogen present in the tropane ring is neutral which may be important for substrate binding and the acid based catalyzed reaction mechanism of BAHD enzymes.

The ability of cocaine synthase to accept other CoA thioesters, albeit at very low rates may explain the trace amounts of other acylated methylecgonine derivatives found in *E. coca* such as hexanoylcocaine methyl ester^{22,23}. In addition we determined that cocaine synthase is specific for catalyzing the esterification of only 3 β -hydroxyl substrates. This specificity is reflected in the supply of methylecgonine via the enzyme methylecgonone reductase (MecgoR). MecgoR only supplies the 3 β -tropane alcohol methylecgonine and is unable to supply the corresponding 3 α -substrate⁹. This explains why tropacocaine is not found in *E. coca*. Specificity for the alcohol containing substrate was also observed for the tropine:acyl-CoA transferase and the pseudotropine:acyl-CoA transferase purified from transformed roots of *D. stramonium* and *Brugmansia candida* x *aurea* hybrid²¹. Lastly, the role of cocaine synthase *in planta* was confirmed using immunoprecipitation followed by protein sequencing.

The distribution of different classes of plant derived alkaloids is depended upon key factors such as substrate availability, tissue specific gene

expression, biosynthetic enzyme localization and transport. In many cases, the interaction of all these factors leads to a complex distribution pattern within the plant. For example, tropane alkaloids produced by members of the Solanaceae largely accumulate in the above ground tissues while the biosynthesis of core metabolites occurs in the roots²⁴. In addition, other alkaloids such as the benzylisoquinoline derivatives in opium poppy accumulate mainly in specialized laticifers found in the vascular tissue while some biosynthetic enzymes are localized to nearby sieve elements²⁵. In *E. coca* the accumulation and biosynthesis of tropane alkaloids occurs within the same tissues. Methylecgonone reductase, the penultimate enzyme in cocaine biosynthesis, is localized to the palisade parenchyma and spongy mesophyll of the leaves⁹. In addition the highest accumulation of tropane alkaloids is also found in these tissues. Moreover the biosynthesis is not dependent upon root tissue²⁶. In this study we have confirmed the tissue localization for the storage of cocaine and other related metabolites in the palisade parenchyma and spongy mesophyll. Cocaine synthase, like MecgoR, is also localized to the same tissues. Cocaine synthase can also be found in the anther tapetum. In addition the enzyme 6 β -hydroxylase (H6H) involved in tropane alkaloid biosynthesis from *Atropa belladonna* is also found in the anther tapetum suggesting a similar biological function of tropane alkaloids in this plant organ²⁷. The anther tapetum is known to accumulate flavonoids as well as anthocyanins which are involved in pollen pigmentation. In order for this pathway to exist, hydroxycinnamoyl-CoA thioesters are required²⁸. This could also supply the required CoA substrates for cocaine synthase. The defensive properties of cocaine have been investigated^{29,30}. However detailed ecological studies on relevant wild relatives are required.

Cocaine synthase is responsible for the production of both cocaine and cinnamoylcocaine in *E. coca*. In the young leaves, cinnamoylcocaine is present in higher amounts and as the leaf matures cocaine levels increase. This is most likely due to the difference in availability of cinnamoyl- and benzoyl-CoA during leaf development. Young coca leaves are high in flavonoids and thus rely upon large pools of

hydroxycinnamoyl-CoA thioesters^{31,32}. As the leaf develops, more of those thioesters can be diverted into the production of benzoyl-CoA³³.

Direct analysis followed by mass spectrometry detection has been found particularly attractive in biomedical and biological applications since it tends to minimize the sample preparation and keeps intact the sample spatial integrity. Particularly, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has been used to map plant metabolites within tissues³⁴⁻³⁶ as well as to monitor specifically cocaine and its metabolites in single intact hair samples from humans for toxicological screening³⁷. Mass spectrometry can detect individual tropane alkaloids based on their molecular weight as opposed to the less specific recognition abilities of the antibodies. To date, no other study using MALDI-MSI of plant leaf tissues has reported differences between the adaxial and abaxial leaf surfaces³⁸⁻⁴¹. The adaxial surface of coca leaves has a thick waxy cuticle containing hentriacontane as one of the major lipid components¹⁰. Wax components in the cuticle of *A. thaliana* are known to decrease the abundance of ions corresponding to flavonoids present within the leaf⁴². In order to remove the wax for further study, the Arabidopsis leaves were dipped in chloroform. In our case, chloroform treatment of leaf stage 2 and 3 leaves did not change the abaxial and adaxial distribution pattern, but it should be noted that the amount of cuticle wax removed was not determined (**Supplementary Fig. 5**). Observed differences in MS signal ratio intensities between both sides of the intact or treated leaves do not take into account possible matrix effects (i.e. ionization suppression or analyte recovery) due to the different wax layer thickness or other endogenous compounds present. Despite the additional experiments to remove surface wax by dipping the leaves into chloroform, tropane alkaloid could still be identified on both sides of the leaves but no relative quantification between abaxial or adaxial leaf surfaces could be achieved. Nevertheless the distribution pattern of tropane alkaloids remains evenly distributed across the leaf in *E. coca* which was also confirmed by recording the MS/MS spectrum and by comparison with authentic standards. In contrast, the tropane alkaloids scopolamine and atropine

are mainly found in the vascular tissues of *D. stramonium* leaves (Solanaceae) when analyzed by indirect desorption electrospray (DESI) ⁴³.

In summary we have determined that the acylation of the 3 β -hydroxyl function of tropane alkaloids in *E. coca* is catalyzed by cocaine synthase, a member of the BAHD acyltransferase family. This is the first report showing that a BAHD member is involved in tropane alkaloid production in plants. We have further been able to show that the distribution of the enzyme is consistent with the hypothesis that tropane alkaloid biosynthesis in *E. coca* occurs in the above ground tissues. This is in contrast to what is known about the tropane alkaloid biosynthetic pathway in members of the Solanaceae. We predict that the dominant esters of tropane alkaloids found in other plant families such as Proteaceae, Brassicaceae, Rhizophoraceae, Convolvulaceae and Solanaceae will be also formed by the action of a BAHD acyltransferase. Isolation of these acyltransferases will be required to determine whether they have been recruited more than once throughout evolutionary history of plants.

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Methods

Chemicals and plant material

Methylecgonine (2-carbomethoxy-3 β -tropine) was purchased from LGC Standards GmbH (Wesel, Germany). Cocaine, cocaine-d₃, cinnamoylcocaine and methylecgonine for MALDI mass spectrometry imaging were provided by Lipomed (Arlesheim, Switzerland). All other chemicals were purchased from Sigma-Aldrich, Carl Roth, or Merck and had the highest available

quality. Water was supplied by a Milli-Q Synthesis System (Millipore). Seeds of *Erythroxylum coca* were obtained from the Botanical Garden in Bonn (Germany). The seeds were removed from the surrounding pulp and germinated in Perlite. The plants were grown at 22 °C under a photoperiod of 12 h light/12 h dark, with a humidity of 65% and 70%, respectively and fertilized once a week with Ferty 3 (15-10-15) and Wuxal Top N (Planta). A voucher specimen was deposited at the Herbarium Haussknecht (JE), Friedrich Schiller University, Jena, Germany. Leaves for MALDI mass spectrometry imaging experiments were collected from *Erythroxylum coca* species at the Botanical Garden in Zurich (Switzerland).

Cloning, heterologous expression, and purification of cocaine synthase and EcBAHD8

Previously, six BAHD acyltransferases were identified from a *E. coca* young leaf λ ZAPII cDNA library ¹⁶. Using these six sequences, a Blast search was performed on an in-house 454 cDNA sequencing database of *E. coca* young leaf tissue yielding two additional sequences, respectively BAHD7 (designated *EcCS*) and *BAHD8* ²⁶. The open reading frames of *EcCS* (Genbank Accession No. KC140149) and *EcBAHD8* (Genbank Accession No. KC140150) were amplified from *E. coca* leaf stage 2 cDNA using primer pairs *EcCS*_EC_Fwd / *EcCS*_EC_Rev and *EcBAHD8*_EC_Fwd / *EcBAHD8*_EC_Rev (**Supplemental Table 2**), respectively and gateway cloned into the *Escherichia coli* expression vector pH9GW as previously described ². The expression vectors were introduced into *E. coli* BL21(DE3) (Invitrogen) and the bacterial culture was grown in LB medium supplemented with 50 μ g/ml kanamycin at 37°C with shaking at 220 rpm until an OD₆₀₀ of 0.4 - 0.5 was reached. Protein expression was induced by addition of 1 mM IPTG with further cultivation at 18 °C for 24 h. The cells were resuspended in 50 mM Bis-tris buffer (pH 8) supplemented with 10% glycerol and 5 mM DTT and disrupted by sonication. The lysate was centrifuged at 15,000 $\times$ g and 4 °C for 15 min and the soluble fractions were tested for enzymatic activity. The assay buffer consisted of 50 mM Bis-Tris propane buffer (pH 8.0) supplemented with 10% (vol/vol) glycerol, 5 mM Tris(2-

carboxyethyl)phosphine hydrochloride (TCEP), 1 mM methylecgonine, 1 mM benzoyl-CoA, and 10 μ L protein extract in a total volume of 100 μ L. The assay was stopped after 2 h with 10 μ L 1 N HCl.

The assays were analyzed by liquid chromatography- ion trap mass spectrometry on an 1100 series equipment HPLC (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) operated in positive ionization mode in the range m/z 50-500. Skimmer voltage, 42.4 V; capillary exit voltage, 110 V; capillary voltage, -4,000 V; nebulizer pressure, 35 psi; drying gas, 9 L min⁻¹; gas temperature, 330 °C. Elution was accomplished using a Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 μ m, Macherey-Nagel, Germany) with a gradient of 0.2% (vol/vol) formic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min at 25 °C as follows: 45-65% B (5 min), 65-100% B (0.1 min), 100% B (1.9 min), 100-45% B (0.1 min), 45 % B (3.9min). Flow coming from the column was diverted in a ratio 4:1 before reaching the ESI unit. Cocaine mass (m/z of 304) and Cinnamoylcocaine (m/z of 330) was monitored, and the area under the product peak was used for activity comparison.

For obtaining sufficient protein for biochemical characterization, the open reading frame of *EcCS* was synthesized as a codon-optimized version designated *EcCSopt* by GenScript (Piscataway, NJ, USA) and supplied in pUC57 vector (GeneScript, USA). *EcCSopt* was amplified from the pUC57 vector using the primer pair EcCSopt_SC_Fwd / EcCSopt_SC_Rev (**Supplemental Table 2**) and gateway cloned into the *Saccharomyces cerevisiae* expression vector pYes-NStrep-GW (*EcBAHD8* was also gateway cloned into pYes-NStrep-GW, using the pDONR207 clone from the initial gateway cloning into pH9GW). The vector pYes-NStrep-GW is a modified pYes-DEST52 (Invitrogen) *S. cerevisiae* expression vector that was built in-house, to facilitate the production of recombinant proteins carrying an N-terminal StrepTagII and a thrombin cleavage site. Furthermore, *EcCSopt* and *EcBAHD8* were amplified using the primer pairs EcCSopt_SC_Fwd / EcCSopt_SC_C-Strep_Rev and EcBAHD8_SC_Fwd / EcBAHD8_SC_C-Strep_Rev (**Supplemental Table 2**) and gateway cloned into the *S.*

cerevisiae expression vector pYes-DEST52 to facilitate the production of recombinant proteins carrying a C-terminal StrepTagII. The expression constructs were introduced into *S. cerevisiae* Inv-Sc1 (Invitrogen) and the protein was expressed as described in the manufacturers' manual. For protein purification the cells were resuspended in 100 mM Tris-HCl (pH 8) supplemented with 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM TCEP and 1 mM phenylmethanesulfonyl fluoride (PMSF) and lysed using a pressure cell homogenizer (Stansted Fluid Power, UK). The lysate was centrifuged at 15,000 $\times$ g and 4 °C for 15 min and the soluble protein was purified using FPLC. After filtration of the sample using a 0.45 μ m-pore-size filter (Millipore), the filtrate was directly loaded onto a StrepTrap HP column (GE Healthcare) using an FPLC machine (ÄKTApurifier, GE Healthcare). Recombinant protein was eluted using 100 mM Tris-HCl (pH 8) supplemented with 150 mM NaCl, 1 mM EDTA, 5 mM TCEP, 1 mM PMSF and 2.5 mM desthiobiotin. Glycerol was added to a final concentration of 10% (vol/vol), and the mix was aliquoted and stored at -20 °C and used directly for enzyme assays. The purity of recombinant protein was evaluated by SDS/PAGE gel electrophoresis followed by colloidal coomassie staining. Protein concentration was measured using the Bradford Protein Assay (Bio-Rad) according to manufacturers' manual. Equivalent amounts of N-terminal and C-terminal StrepTagII-tagged recombinant protein were tested for enzyme activity, but no significant difference was observed. N-terminal StrepTagII-tagged protein expressed in *S. cerevisiae* was used for enzyme kinetics.

Extraction of protein from E. coca tissues

Fresh *E. coca* tissues were harvested and ground using a mortar and pestle precooled with liquid nitrogen. The plant powder was mixed in a ratio of 1:5 with 100mM Tris-HCl pH 8 supplemented with 10% (vol/vol) glycerol, 2% (wt/vol) polyvinylpyrrolidone (PVPP), 50 mM Na₂S₂O₅, 5 mM DTT and 1 mM PMSF. The resulting emulsion was mixed and incubated for 10 min on ice followed by centrifugation at 16,000 $\times$ g at 4 °C for 10 min. The supernatant is referred to as the plant extract, and was used

directly for immunoblotting analysis. For plant activity assays, the plant extracts were desalted into 100 mM TrisHCl pH 8 supplemented with 10% (vol/vol) glycerol and 5 mM TCEP using Illustra Nap-5 columns (GE Healthcare) according to the manufacturers' instructions. Protein concentration was measured using the Bradford Protein Assay (Bio-Rad) according to the manufacturers' manual.

Synthesis of cinnamoyl-CoA

The synthesis of the activated cinnamic acid was performed as described ⁴⁴ with some modifications. 50 mmol cinnamic acid was added to a solution of 120 mmol carbonyldiimidazole in tetrahydrofuran. The reaction mixture was stirred for 5 h at reflux. After cooling to room temperature any solids were filtered off. The solvent was removed at a rotary evaporator. The pale yellow solid was washed with 10 mL water and dried *in vacuo*. 0.16 mmol of activated cinnamic acid was dissolved in 700 μ L 50mM NaHCO₃ and added slowly to a solution of 0.13 mmol CoA in 500 μ L of 50mM NaHCO₃ at 3°C. Acetone was added until the precipitate dissolved completely. The mixture was stirred 24 h at 3°C. Afterwards the acetone was removed with N₂. The CoA ester was purified as described in ¹ and fractions were analysed by HPLC UV. The absorbtion at 260 nm was used for quantification using coenzyme A tritium salt. The fraction with with absorbtion at 260nm (6.3 min) was further analyzed by liquid chromatography - ion trap mass spectrometry on an 1100 series equipment HPLC (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) operated in negative ionization mode in the range m/z 100-1000. Skimmer voltage, 42.4 V; capillary exit voltage, 123.7 V; capillary voltage, -4,500 V; nebulizer pressure, 35 psi; drying gas, 10 L min⁻¹; gas temperature, 330 °C. Elution was accomplished using a Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 μ m, Macherey-Nagel, Germany) with a gradient of 20 mM (vol/vol) ammonium acetate (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min at 25 °C as follows: 15-29% B (7 min), 29-90% B (1 min), 90% B (2 min), 90-15% B (0.1 min), 15 % B (4.9min). Flow coming from the column was

diverted in a ratio 4:1 before reaching the ESI unit.

Measurement of methylecgonine, cinnamoylcocaine and cocaine in plant tissue

E. coca leaves of all three leaf stages (L1, L2, L3), flower, stem and roots were ground to a fine powder in liquid N₂. Tropane alkaloids were extracted 1:10 (w/v) using 0.1% (v/v) formic acid, 30% (v/v) methanol at room temperature for 15 min. Samples were centrifuged at 4000 x g for 15 min. The supernatant was filtered using 0.45 μ m syringe filters and adjusted to pH 8 with saturated sodium carbonate solution followed by reextraction with three times chloroform. Phase separation was achieved using Chromabond PTS columns (Macherey-Nagel, Germany). The organic phase was vacuum dried and solved in water for analysis. Measurements were taken for six replicates of each sample. Analysis was done as described in section **Enzyme assays for kinetic analysis of enzymes and plant activity determination** using authentic standards of methylecgonine, cocaine and cinnamoylcocaine.

EcCS and EcBAHD8 localization in E. coca Organs

Protein from different plant organs was extracted as described above. Equal amounts (15 μ g) of protein were immunoblotted as described previously ⁹ with the following exceptions. After blocking, the membranes were incubated with 1:1000 anti-EcCS and 1:5000 anti-rabbit HRP-conjugated antibodies (Sigma-Aldrich) in blocking solution. For immunohistochemistry, selected fresh tissues were harvested and fixed for 4 h at room temperature in 50% (vol/vol) ethanol, 5% (vol/vol) acetic acid and 3.7% (vol/vol) formaldehyde using vacuum infiltration. Samples were rinsed 3 times for 10 min in PBS (2.7 mM KCl, 137 mM NaCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, adjusted to pH 7.4 with 1N KOH). Samples were dehydrated in a ascending series of ethanol solutions (vol/vol) (10%, 30%, 50%, 70%, 90%) each time for 1h. Samples were left in 96% (vol/vol) ethanol overnight, followed by an ascending series in Roti-Histol (Carl Roth), 25% (vol/vol), 50% (vol/vol), 75% (vol/vol), and 100% for 1 h each. The following steps were performed at 60 °C. Roti-Histol (Carl Roth) was exchanged with liquid Paraplast X-Tra (Carl Roth) over a course of 4 d by replacing half of the

solution with liquid Paraplast X-Tra every 12 h. Remaining Roti-Histol was allowed to evaporate in an open container at 60 °C for 5 h. Single plant organs were embedded in aluminum molds and left to settle at room temperature for 30 min, before being transferred to 4 °C for storage. Samples were cut on a rotary microtome to obtain 10 µm slices which were adhered to polylysine coated slides (Thermo Scientific) at 42 °C overnight. To prepare for antibody treatment, slides were incubated in Roticlear (Carl Roth) and rehydrated by a descending ethanol series (2x 100%, 95% (vol/vol), 70% (vol/vol), 50% (vol/vol), 30% (vol/vol), 15% (vol/vol) and 2x H₂O) for 2 min at each concentration. Endogenous peroxidases were blocked with 3% (vol/vol) H₂O₂ in PBS for 30 min. Slides were washed twice with PBS and once with PBT (PBS supplemented with 0.1% (vol/vol) Tween 20) for 5 minutes each, and blocked overnight with 1% (wt/vol) BSA in PBT at 4 °C.

For localization of EcCS slides were incubated with 1:100 anti-EcCS antibody. For localization of cocaine slides were incubated with 1:100 sheep anti-benzoylcegonine antibodies (RayBiotech, Norcross, USA). Anti-rabbit HRP-conjugated antibody (Sigma-Aldrich) or 1:100 anti-sheep HRP-conjugated antibody (RayBiotech, Norcross, USA) was used as secondary antibody. For fluorescent staining, TSA Kit #24 (Invitrogen) was used according to the manufacturers' manual. For cocaine localization slides were imaged using a Zeiss LSM510 confocal microscope and a 20x objective lens (Plan-Apochromat 20x/0.8 M27, Carl Zeiss). TSA fluorescence was excited at 543 nm and detected using a BP 585–615 filter. Plant autofluorescence was excited at 488 nm and detected using LP 505nm filter. For EcCS localization slides were imaged using a Zeiss LSM710 confocal microscope and a 20x objective lens (EC Plan-Neofluar 20x/0.5 M27, Carl Zeiss). TSA fluorescence was excited at 561 nm and detected using 585–614 nm lambda channels. Plant auto fluorescence was excited at 488 nm and detected using 495-534 nm lambda channels. Overview pictures were obtained, by taking tile pictures of whole tissue sections. TSA fluorescence and plant auto fluorescence were imaged simultaneously, overlaid using ImageJ⁴⁵ and transferred to

Illustrator CS6 (Adobe Systems) without further manipulation.

EcCS Antibody Production

Purified recombinant EcCS protein carrying an N-terminal StrepTagII produced in *S. cerevisiae* as described above, was used to produce polyclonal antibodies in rabbits. Antibodies were affinity-purified using epoxy-activated Sepharose conjugated to the same recombinant protein that was used for immunization (Davids Biotechnologie, Regensburg, Germany). Specificity of the purified antibodies to EcCS in comparison to other *E. coca* BAHD proteins was assessed by immunoblotting of recombinant proteins expressed in *E. coli* using the pH9GW vector as described above (**Supplemental Fig. 2b**).

Immunoprecipitation

Protein from leaf stage 2 leaves of *E. coca* was extracted as described in above. Protein was desalted into immunoprecipitation buffer (50 mM BisTris pH 8 supplemented with 10% (vol/vol) glycerol, 1 mM TCEP, 100 mM NaCl, 1 mM PMSF and 0.5% (vol/vol) NP-40). Protein concentration was measured as described earlier. 100 µl Protein A coupled agarose beads (GE Healthcare) were prepared as described in the manual. 500 ng protein were adjusted to 700 µl with immunoprecipitation buffer. 5 µg of anti-EcCS antibody and 20 µl of agarose bead slurry were added to the protein sample and incubated at 4 °C for one hour. The immunoprecipitate, represented by the agarose beads, was separated from the supernatant by centrifugation at 12000 x g for 20 s at 4 °C. The supernatant was kept as the IP supernatant sample. The immunoprecipitate was washed three times with 1ml of immunoprecipitation buffer, and the wash solutions were discarded. Enzyme assays on the immunoprecipitate and supernatant were analyzed on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 µm) (Agilent). Separation was achieved in 7 min, at 20 °C and a flow rate of 800 µL/min, using formic acid 0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7

min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 μ L; curtain gas, 30 psi; turbo heater temperature, 700 $^{\circ}$ C; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 $\rightarrow$ 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V). Quantification was based on a standard curve of authentic cocaine. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

Cocaine Synthase Substrate Specificity

Enzyme assays were performed as described in section **Enzyme assays for kinetic analysis of enzymes and plant activity determination** with the exception that various coenzyme A esters were used for the esterification reaction with methylecgonine. Besides benzoyl and cinnamoyl coenzyme A, acetyl, acetoacyl, coumaroyl, hexanoyl and malonyl coenzyme A were tested. In lack of access to the corresponding esters, the product ion was predicted and the peak area of the product peaks was used for comparison with the peak area of cocaine, assuming that ionization of the molecules as well as the response of the machine are similar for the ester products. Quantification was based on an authentic standard curve for cocaine. The relative activities compared to cocaine are only based upon peak areas. Atropine served as internal standard and enzyme assays (100 μ L) were diluted with 900 μ L of methanol, spiked with internal standard before injection.

Analysis was carried out on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 μ m) (Agilent). Separation was achieved in 7 min, at 20 $^{\circ}$ C and a flow rate of 800 μ L/min, using formic acid 0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7 min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 μ L; curtain gas, 30 psi; turbo heater temperature, 700 $^{\circ}$ C; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes

were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 $\rightarrow$ 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V); cinnamoylcocaine m/z 330.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); methylecgonine m/z 200.2 $\rightarrow$ 182.3 (CE 23V; DP 31V); acetyl product m/z 242.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); acetoacyl product m/z 284.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); coumaroyl product m/z 346.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); hexanoyl product m/z 298.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); malonyl product m/z 286.3 $\rightarrow$ 182.3 (CE 26V; DP 45V); atropine m/z 290.1 $\rightarrow$ 124.1 (CE 31V; DP 51V) Quantification was based on a standard curve of authentic cocaine and cinnamoylcocaine, taking the signal from the internal standard into account. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

Size-exclusion chromatography

Protein size of EcCS was determined using an ÄKTApurifier (GE Healthcare) equipped with a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare). Recombinant protein was expressed in *S. cerevisiae* and purified as described earlier. The running buffer consisted of 50 mM BisTris pH 8.0 supplemented with 150 mM NaCl. The column was calibrated using the Gel Filtration LMW Calibration Kit (GE Healthcare) as described in the manufacturers' instructions. Samples were loaded at a flow rate of 0.5 mL/min. Protein was eluted at a flow rate of 1 mL/min for 1.5 CV while collecting fractions of 6 mL.

Synthesis of $^{13}\text{C}_7$ -Cocaine

^{13}C -benzoyl coenzyme A was prepared as described ⁴ with the exception that benzoic acid was isotopical labeled. $^{13}\text{C}_7$ benzoic acid was obtained from Isotec (Miamisburg, OH, USA). An enzymatic assay (200 μ L) containing 50 mM potassium phosphate buffer pH 8 supplemented with 1 mM TCEP, 1 mg/mL BSA, 0.1 mM $^{13}\text{C}_7$ -benzoyl-CoA, 0.5 mM methylecgonine and 8 μ g purified EcCS was left to react overnight at room temperature. Another 8 μ g purified EcCS was added and left to react at room temperature for 12 h. The solution was basified using 20 μ L 1 N NaOH and extracted 3 times with 500 μ L chloroform. The chloroform phase was dried down under nitrogen flow and resuspended in 200 μ L 10% (vol/vol) ethanol supplemented with

0.1% (vol/vol) formic acid. To determine the concentration for use as internal standard, dilutions of the product were analyzed on an API 3200 tandem mass spectrometer (Applied Biosystems) as described below.

Enzyme Assays for Kinetic Analysis of Enzymes and Plant Activity Determination.

Protein concentration and incubation parameters were chosen so that the reaction velocity was linear in respect to enzyme concentration and incubation time for all enzyme assays. Standard assays contained 50 mM glycine-NaOH pH 9.4, 1 mg/mL BSA, 1 mM TCEP, 1 mM methylecgonine, 1 mM of benzoyl- or cinnamoyl-CoA, and the enzyme preparation. All reactions were carried out at 20 °C in a PCR cycler Primus 96 plus (MWG Biotech, Ebersberg, Germany). For determination of the pH optimum 50 mM glycine-NaOH buffer covering the range from pH 8.6 to 10.4 was used. Enzyme and substrate concentrations were varied during characterization process. For kinetic assays, the co-substrate concentration was held constant. Reactions (50 µL) were stopped after 10 min by adding 5 µL 1 N HCl and 5 µL of 2.5 µM ¹³C₇-cocaine was added as internal standard. 100 µL chloroform were added to the assays and shaken in a paint shaker for 3 min, to remove the protein from the assay. The aqueous phase was transferred into fresh HPLC vials and subjected to LC-MS/MS analysis.

Analysis was carried out on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 µm) (Agilent). Separation was achieved in 7 min, at 20 °C and a flow rate of 800 µL/min, using formic acid 0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7 min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 µL; curtain gas, 30 psi; turbo heater temperature, 700 °C; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 → 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V); cinnamoylcocaine m/z 330.3 → 182.3 (CE

26V; DP 45V); methylecgonine m/z 200.2 → 182.3 (CE 23V; DP 31V); ¹³C₇-cocaine m/z 311.3 → 182.3 (CE 26V; DP 45V). Quantification was based on a standard curve of authentic cocaine and cinnamoylcocaine, taking the signal from the internal standard into account. Both Q1 and Q3 quadrupoles were maintained at unit resolution. For kinetic analysis of EcBAHD8 with cinnamoyl-CoA and methylecgonine, the analysis was carried out as described above except for the following changes. An API5000 tandem mass spectrometer (Applied Biosystems) was used and all chromatographic and mass spectrometer parameters were the same, except for the declustering potential settings which were as follows: cocaine 75 V; cinnamoylcocaine 75V; methylecgonine 61V; ¹³C₇-cocaine 75V. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Calculations and fitting of kinetic curves was performed using Origin 8.5 (OriginLab, USA).

Quantitative real-time PCR analysis

For relative quantification experiments were performed as described ⁵. Primer pairs targeting the *EcCS* and *EcBAHD8* transcripts EcCS_qPCR_Fwd / EcCS_qPCR_Rev and EcBAHD8_qPCR_Fwd / EcBAHD8_qPCR_Rev (**Supplemental Table 2**) were designed. Standard curve analysis showed a PCR efficiency of 95.5% and 88.3% and R² values of 0.9983 and 0.9992 for EcCS and EcBAHD8 primer pairs, respectively. Expression of the genes was normalized to gene 6409 and gene 10131 ⁵ expression using qBase version 1.3.5 ⁶.

For absolute quantification experiments, pDONR207 plasmids harbouring *EcCS* or *EcBAHD8* obtained during the cloning procedure, were quantified using NanoDrop 2000c (NanoDrop Technologies, Wilmington, USA). Standard curves of plasmids ranging from 10 to 10⁹ plasmids per assay were run in parallel to qPCR assays as described ⁵. Standard curve analysis showed a PCR efficiency of 78.8% and 85.8% and R² values of 1.000 and 0.998 for EcCS and EcBAHD8 primer pairs, respectively. The data was normalized to the input amount of the total RNA to the original cDNA synthesis reaction.

Phylogenetic Analysis

Analysis of protein sequences (see **Supplemental Table 1** for accession numbers) was performed as previously described¹⁶. The following enzymes and their corresponding genbank accession numbers were added to the analysis EcCS (cocaine synthase) - KC140149 and EcBAHD8 - KC140150.

MALDI Imaging

Standard and matrix solutions: α -cyano-4-hydroxycinnamic acid (CHCA) was dissolved at a concentration of 10 mg/mL in MeOH:H₂O:HCOOH (75:25:0.1, vol/vol/vol). Deuterated cocaine (cocaine-d₃) was added to the matrix solution (final concentration of 200 ng/mL) and sprayed simultaneously with the matrix. Matrix solutions were kept in amber glass bottles to prevent their degradation by UV and stored at 4°C between uses.

E. coca leaf samples were collected at three different stages of maturity: leaf stage 1; leaf stage 2 and leaf stage 3. After being cut at the basis of the stem, samples were then placed in-between two aluminum foils (to maintain their flat shape), and immersed immediately in liquid nitrogen for flash freezing. Additionally, a set of leaf stage 2 and leaf stage 3 leaves were dipped in chloroform for 5 seconds prior flash freezing to remove waxy cuticle. Frozen samples were then stored at -80°C prior to the analysis.

After drying in a desiccator under vacuum for 20 min at room temperature, intact leaf samples were mounted onto the stainless steel MALDI plate (OPTI TOF 384 well Insert - 123 x 81 mm or OPTI TOF 192 well insert - 44 x 44 mm, AB SCIEX, Concord, ON, Canada) using a double-sided adhesive tape (Plasto, Chenove, France). Two intact leaves from the same maturity stage were placed on the MALDI plate in order to analyze both sides of the leaves in the same run. Approximately 12 mL of CHCA solution were manually sprayed using a Custom Micron CM-C Plus airbrush 0.18 mm (ANEST IWATA Corporation, Yokohama, Japan) held at a distance of 20 cm from the plate. N₂ was used as the nebulizer gas (purity > 99.995%, Messer, Switzerland) and operated at a constant pressure of 0.5 bars. For the semi-automated spraying, the airbrush was fixed on a motorized XY two-axis stage (Zaber Technologies Inc., Vancouver, BC,

Canada) to control the sprayer motion and speed. The sprayer was placed at a distance of 25 cm from the target plate and moved linearly at 15 mm/s. Thirty layers of CHCA matrix solution containing the internal standard (solution of 200 ng/mL) were applied.

Acquisitions were performed on a triple quadrupole linear ion trap mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with a MALDI source and a frequency-tripled Nd:YAG laser 355 nm (elliptical beam shape of 100x200 μ m). MS and MS/MS images were acquired in positive ionization mode. General operating conditions were: data acquisition mode = line scan in rastering mode, repetition rate laser = 1'000 Hz; laser energy = 60 μ J; MALDI source and q₀ region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q₂ = 2.4 10⁻⁵ Torr (nitrogen); declustering potential = 70 V; entrance potential = 10 V; quadrupole resolution was set to unit for Q₁ and Q₃.

SRM data were acquired by monitoring simultaneously the following transitions: m/z 304 > m/z 182 for cocaine (CE = 35 eV), m/z 307 > m/z 185 for cocaine-d₃ (CE = 35 eV), m/z 330 > m/z 182 for cinnamoylcocaine (CE = 40 eV) and m/z 200 > m/z 182 for methylecgonine (CE = 40 eV). The dwell time was set to 500 ms (TST = 2.020 s) and the distance between two line scans was of 1 mm (rastering speed of 0.5 mm/s), resulting in MALDI-SRM/MS-based images with pixels of 1x1 mm for the four compounds. To generate MALDI-SRM/MS images at higher resolution (*i.e.* pixels of 50x50 μ m²), the plate speed was kept constant at 0.5 mm/s, the dwell time was decreased to 20 ms, and the distance between 2 line scans was set to 50 μ m (oversampling technique, see results and discussion part).

MS/MS spectra in the enhanced product ion (EPI) scan mode were acquired at a plate speed of 1 mm/s and the distance between two line scans was of 2 mm (pixels of 2 x 2 mm²). The conditions for each compounds were: i) for cocaine: precursor ion = m/z 304; CE = 35 eV, scan range = m/z 80 - 310, TST = 1.991 s ; ii) for cinnamoylcocaine: precursor ion = m/z 330; CE = 35 eV, scan range = m/z 80 - 340, TST = 2.051 s; iii) for methylecgonine: precursor ion = m/z 200; CE = 45 eV, scan range = m/z 80 - 210, TST = 1.991 s. Reference spectra were acquired from a

MALDI spot where 1 μ L of a standard solution with the 3 analytes at a concentration of 100 ng/mL mixed 1:1 (v:v) with CHCA (10 mg/mL) was spotted onto the target plate. Spectra for blank samples and for structural confirmation at the different maturation stages were generated by summing 6 vertical adjacent pixels over selected regions.

The MALDI source and its laser were controlled using a custom M3Q Server software based on a LabView platform (AB SCIEX). Analyst 1.5 software (AB SCIEX) was used for mass spectrometer control and for data collection. PeakView software (v.1.0.0.3, AB SCIEX) was used for raw data processing. A dedicated script was provided by Eva Duchoslav (AB SCIEX) to convert raw MS data files into an .img file that is compatible with the TissueView software (v.1.0, AB SCIEX) for MS and MS/MS images generation and processing.

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Supplementary Material

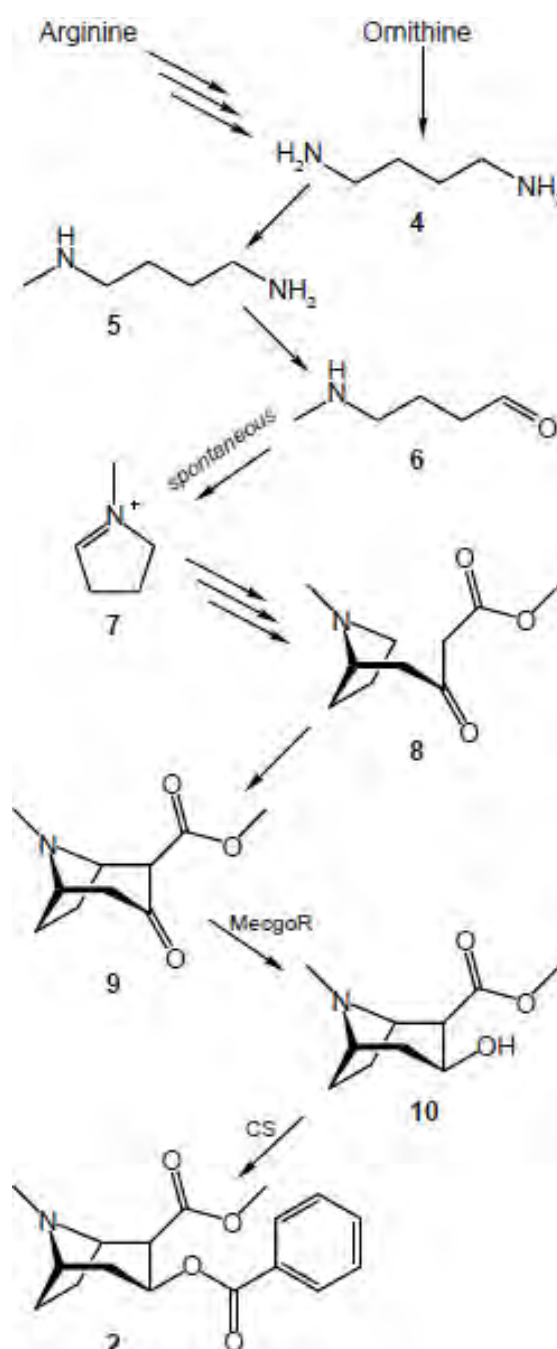
Supplemental Table 1 | Details of the abbreviated BAHD acyltransferases used for Fig. 3. Included in the table are which major acyl CoA unit is known, what the major class of products are formed and which species the particular acyltransferase was identified in.

Acyltransferase Name	NCBI genbank protein ID no.	Major Acyl CoA donor	Major product(s) formed	Species
DAT	AAC99311	Acetyl	Vindoline	<i>C. roseus</i>
MAT	AA013736		Minovincinine	<i>C. roseus</i>
CbBEAT	AAC18062	Acetyl	Benzyl acetate	<i>C. breweri</i>
CbBEBT	AAN09796	Benzoyl	Benzyl benzoate	<i>C. breweri</i>
NtBEBT	AAN09798	Benzoyl	Benzyl benzoate	<i>N. tabacum</i>
CHAT	AAN09797	Acetyl	(Z)-3-hexen-1-yl acetate	<i>A. thaliana</i>
BPBT	AAU06226	Benzoyl	Benzyl benzoate/ Phenethyl benzoate	<i>Petunia x hybrida</i>
HCBT	CAB06430	Hydroxy-cinnamoyl/ Benzoyl	Dianthramides	<i>D. caryophyllus</i>
SAAT	AAG13130	Acetyl	Medium chain aliphatic and benzyl esters	<i>Fragaria x ananassa</i>
Vinorine synthase	CAD89104	Acetyl	Vinorine	<i>R. serpentina</i>
SalAT	AAK73661	Acetyl	Thebaine	<i>P. somniferum</i>
Ss5MaT2	AAR26385	Malonyl	Anthocyanins	<i>S. splendens</i>
Gt5AT	BAA74428	Hydroxy-cinnamoyl	Anthocyanins	<i>G. triflora</i>
Dm3MAT2	AAQ63616	Malonyl	Anthocyanins	<i>Chrysanthemum x morifolium</i>
Dm3MAT1	AAQ63615	Malonyl	Anthocyanins	<i>Chrysanthemum x morifolium</i>
Dv3MAT	AA012206	Malonyl	Anthocyanins	<i>D. variabilis</i>
Vh3MAT1	AAS77402	Malonyl	Quercetin 3-O-6"- O-malonylglucoside	<i>Verbena x hybrida</i>
Lp3MAT1	AAS77404	Malonyl	Quercetin 3-O-6"- O-malonylglucoside	<i>L. purpureum</i>
Ss5MaT1	AAL50566	Malonyl	Anthocyanins	<i>S. splendens</i>
Sc3MaT	AA038058	Malonyl	Anthocyanins	<i>P. cruenta</i>
Pf5MaT	AAL50565	Malonyl	Anthocyanins	<i>P. frutescens</i>
Pf3AT	BAA93475	Hydroxy-cinnamoyl	Anthocyanins	<i>P. frutescens</i>
ACT	AA073071	Hydroxy-cinnamoyl	Hydroxy-cinnamoyl agamatine derivatives	<i>H. vulgare</i>
NtHCT	CAD47830	Hydroxy-cinnamoyl	Chlorogenic acid and derivatives	<i>N. tabacum</i>
AtHCT	NP_199704	Hydroxy-cinnamoyl	Chlorogenic acid and derivatives	<i>A. thaliana</i>
AsHHT1	BAC78633	Hydroxy-cinnamoyl	Avenanthramides	<i>A. sativa</i>
NtHQT	CAE46932	Hydroxy-cinnamoyl	Chlorogenic acid	<i>N. tabacum</i>
CmAAT(1-4)	CAA94432 AAL77060 AAW51125 AAW51126	Medium chain aliphatic	Medium chain and hydroxycinnamoyl acyl esters	<i>C. melo</i>
Pun1	AAV66311	unknown	Capsaicin pathway	<i>C. annum</i>
DBNTBT	AAM75818	Benzoyl	2'-deoxytaxol	<i>T. canadensis</i>
BAPT	AAL92459	B-phenylalanoyl	N-debenzoyl-2'- deoxytaxol	<i>T. cuspidata</i>
DBBT	Q9FPW3	Benzoyl	7,13-diacetylbaccatin III	<i>T. cuspidata</i>

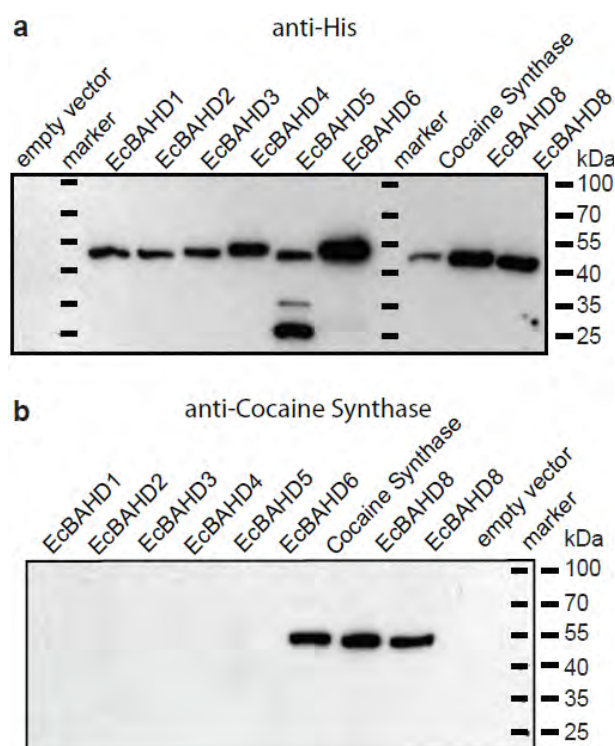
TAT	AAF34254	Acetyl	taxa-4(20),11(12)-dien-5a-yl acetate	<i>T. cuspidata</i>
DBAT	AAF27621	Acetyl	Baccatin III	<i>T. cuspidata</i>
Cer2	AAM64817	Acetyl?	C32 epicuticular waxes	<i>A. thaliana</i>
Glossy2	CAA61258	Acetyl?	C32 epicuticular waxes	<i>Z. mays</i>
AMAT	AAW22989	Anthaniloyle	Methyl anthranilate	<i>V. labrusca</i>
NtMAT1	BAD93691	Malonyl	Flavonoid and naphthol glucosides	<i>N. tabacum</i>
MpAAT1	AAU14879	Short to medium chain aliphatic	Short to medium chain aliphatic volatile esters	<i>Malus x domestica</i>
HMT/HLT	BAD89275	Tigloyl	Quinolizidine alkaloids	<i>L. albus</i>
RhAAT1	AAW31948	Acetyl	Geranyl acetate and other volatile esters	<i>Rosa hybrid cultivar</i>
VAAT	AF193790_1	Short to medium chain aliphatic	A wide range of volatile esters in the fruit	<i>F. vesca</i>
BanAAT	AX025506	Short to medium chain aliphatic	A wide range of volatile esters in the fruit	<i>Musa acuminata</i> × <i>balbisiana</i>
At5MAT	NM_113880	Malonyl	Cyanidin anthocyanin	<i>A. thaliana</i>
AtSHT	NP_179497	Hydroxycinnamoyl	Acyated polyamines	<i>A. thaliana</i>
CsHCT	ACF37072	Hydroxycinnamoyl	Quinate esters	<i>Cynara cardunculus</i>
AtHHT1	NP_851111	Feruloyl	Acyated palmitate esters	<i>A. thaliana</i>
OsMAT1	NP_001046857	Malonyl	Acyated flavonoids	<i>O. sativa</i>
TpHCT2	ACI16631	Hydroxycinnamoyl	Malate esters	<i>T. pratense</i>
AtACT	NP_200924	Hydroxycinnamoyl	Acyated anthocyanin	<i>A. thaliana</i>
AtSDT	NP_179932	Sinapoyl	Spermidine amides	<i>A. thaliana</i>
AtSCT	AAP81804	Coumaroyl	Spermidine amides	<i>A. thaliana</i>
GmIF7MaT	NP_001237760	Malonyl	Isoflavone esters	<i>G. max</i>
CbRAS	CAK55166	Hydroxycinnamoyl	Rosmarinic acid	<i>C. blumei</i>
CcHQT	ABO77956	Hydroxycinnamoyl	Chlorogenic acid	<i>C. canephora</i>
EcBAHD1	JQ413184	unknown	unknown	<i>E. coca</i>
EcBAHD2	JQ413185	unknown	unknown	<i>E. coca</i>
EcBAHD3	JQ413186	unknown	unknown	<i>E. coca</i>
EcHQT	JQ413187	Hydroxycinnamoyl	Hydroxycinnamoyl quinate esters	<i>E. coca</i>
EcBAHD5	JQ413188	unknown	unknown	<i>E. coca</i>
EcBAHD6	JQ413189	unknown	unknown	<i>E. coca</i>
Cocaine synthase	KC140149	Benzoyl Cinnamoyl	Cocaine Cinnamoylcocaine	<i>E. coca</i>
EcBAHD8	KC140150	Benzoyl Cinnamoyl	Cocaine Cinnamoylcocaine	<i>E. coca</i>

Supplemental Table 2 | Primer sequences used for cocaine synthase and EcBAHD8

Primer name	Primer sequence (5' → 3')
EcCS_EC_Fwd	CTGGTTCCGCGTGGTTCCATGGAAATGTCTAAAAAGAAGCTAGAAATCA
EcCS_EC_Rev	CAAGAAAGCTGGGTCTGAATCACATGCGACAATATGCTG
EcCSopt_SC_Fwd	CTGGTTCCGCGTGGTTCCAAAAATGGAAATGTCTA
EcCSopt_SC_Rev	CAAGAAAGCTGGGTCTTACATACGACAATAAGCA
EcCSopt_SC_C-Strep_Rev	CAAGAAAGCTGGGTCTTATTTTTTCGAACTGCGGGTGGCTCCAAGCGCTCATA CGACAATAAGCAGCCAGAACAG
EcCS_qPCR_Fwd	ggcaattcaacttacactcttcaactg
EcCS_qPCR_Rev	AACAAACCTTCTCTTAACGAACTCTCC
EcBAHD8_EC_Fwd	CTGGTTCCGCGTGGTTCCATGGAAATGCCTAACTTTCTGGAAATG
EcBAHD8_EC_Rev	CAAGAAAGCTGGGTCTCACATGCGAGAATATGCTGCAAG
EcBAHD8_SC_Fwd	CTGGTTCCGCGTGGTTCCAAAAATGGAAATGCCTAACTTTCTGGAAATG
EcBAHD8_SC_C-Strep_Rev	CAAGAAAGCTGGGTCTTATTTTTTCGAACTGCGGGTGGCTCCAAGCGCTCATG CGAGAATATGCTGCAAG
EcBAHD8_qPCR_Fwd	GCCGATTTCTTGAGCCACTTCTG
EcBAHD8_qPCR_Rev	GTTACTACTTTGCTGCTGCCTTCC



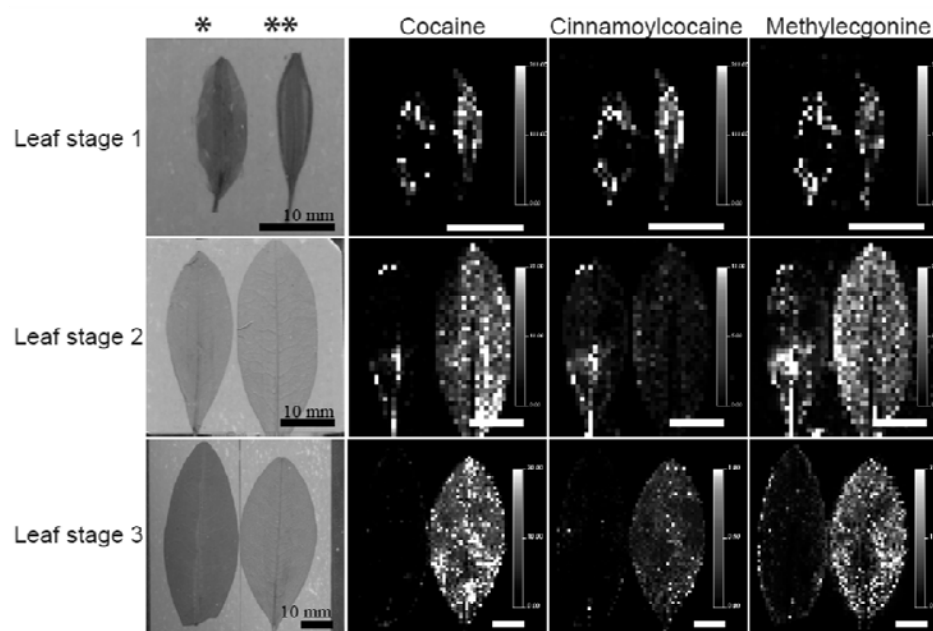
Supplemental Figure 1 | Hypothetical cocaine biosynthesis pathway. The pathway recruits the primary metabolites ornithine and arginine, to form putrescine (4) which is methylated to the first dedicated intermediate *N*-methylputrescine (5) which is subsequently oxidized to 4-methylaminobutanal (6). Spontaneous cyclisation of (6) leads the formation of the *N*-methyl-Δ¹-pyrrolinium cation (7). Further biosynthetic steps proceed through an oxobutanoic acid intermediate (8) to methylecgonone (9), which is reduced to methylecgonine (10) by the action of methylecgonone reductase. Cocaine (2) is formed by esterification of (10) with benzoyl coenzyme A through the enzyme cocaine synthase.



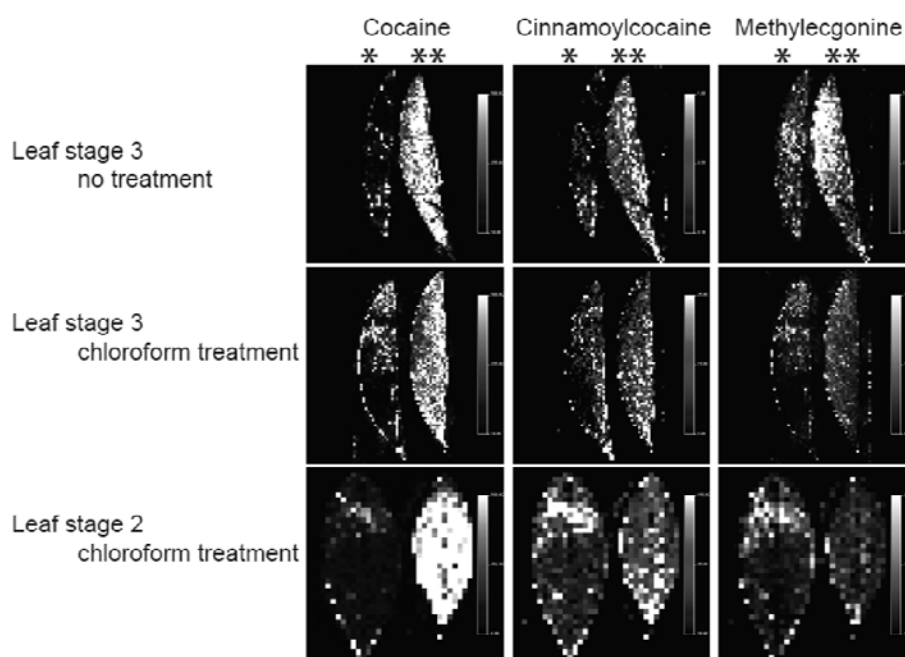
Supplemental Figure 2 | Immunoblot analysis to assess the potential cross-reactivity of the polyclonal anti-Cocaine Synthase antibody with other BAHD acyltransferase proteins from *E. coca*. Acyltransferases isolated from *E. coca* were heterologously expressed in *E. coli* carrying an 9x His-tag. Crude *E. coli* protein extracts were subject to SDS/PAGE and gels were blotted onto filters. The filters were first probed with anti-His antibodies (a) or anti-cocaine synthase antibodies (b), followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Anti-His antibodies were used to prove that recombinant acyltransferases are present. Polyclonal antibodies recognize cocaine synthase and EcBAHD8. Bands were visualized using chemiluminescence.

EcCS	ATGGAAATGCTCTAAAGCAAGCTGAAATCATCTTAAGAAAAACATTAACCATCTCTTCTACCCCTCA	71
EcCSopt	ATGGAAATGCTCTAA AA AAGCT GAAAT AT T G AAAAC ATTAACCATC TCTTCTAC CC CA	71
EcCS	ACATCTCAAACTTTGACCTTTCTTTTGGGACGAACCCCTCCCTCCGATTATGGCACTATATTTTCT	142
EcCSopt	ACATCTCAAACTTTGACCTTTCTTTTGGGAGAACCCCTCCCTCCGATTATGGTACCATATTTTCT	142
EcCS	TCTACCAACCAATGGCTCCAAAAATGATGATGATGAAGCTCTCTCATTTTCTTCCAAAGCTCCTCTTC	213
EcCSopt	TCTACCAACCAATGGCTCTAAAAATGATGATGATGAAGCTCTCTCATTTTCTTCCAAAGCTCCTCTTC	213
EcCS	CTACCAATTCCTCTCCAAAACTGATTCACTACTATCCATTGGCAGGAGACTCAAGATGATGGTAC	284
EcCSopt	CTACCAATTCCTCTCTCAAACTGATTCACTATATCCATTGGCTGGTCCCTCAAGATGATGGTAC	284
EcCS	AGCGTTGACCTCAATGATGAAGCGCTTATTTTGTGAAGCTCGATTGATTCACACTCTCTACCTTGC	355
EcCSopt	AGCGTTGACCTCAATGATGAAGCGCTTATTTTGTGAAGCTCGATTGATTCACACTCTCTACCTTGC	355
EcCS	TAAACATCCGATGCTGATTTCTTGAGCCATCTTGTCCGCTTTGGATTCTAAACTTCCCTCA	426
EcCSopt	TAAACATCCGATGCTGATTTCTTGAGCCATCTTGTCCGCTTTGGATTCTAAACTTCCCTCA	426
EcCS	GGTGTATGTTGGCAATTCACACTTACACTCTTCAACTCGGAGGATAGCAATTAGTGTGAGCCCTCGCA	497
EcCSopt	GGTGTATGTTGGCAATTCACACTTACACTCTTCAACTCGGAGGATAGCAATTAGTGTGAGCCCTCGCA	497
EcCS	TAAAAATGCGATGCTTCTCGCTGACCTTTGTCCAGTTGGGCTCATGACTACATCGGGAAG	568
EcCSopt	TAAAAATGCGATGCTTCTCGCTGACCTTTGTCCAGTTGGGCTCATGACTACATCGGGAAG	568
EcCS	TAGCCCTCAAACTTATTTCTGGAACCATCTCTCCACCACGCTCTTACGTAAAGCCCATGCTCA	639
EcCSopt	TAGCCCTCAAACTTATTTCTGGAACCATCTCTCTCCACCACGCTCTTACGTAAAGCCCATGCTCA	639
EcCS	ACGAAATACCGGGAGTTGGTTAAGAGAGCTTTGTTTCCCGCTCAAAAATGCTCAACTCAGSAC	710
EcCSopt	ACGAAATACCGGGAGTTGGTTAAGAGAGCTTTGTTTCCCGCTCAAAAATGCTCAACTCAGSAC	710
EcCS	AAAAACACCGGCTCTCTCCACAGGAAAGCAACATATGATGATGCTGATTGGTGATGGCGTTAT	781
EcCSopt	AAAAACACCGGCTCTCTCCACAGGTAACAACATATGATGATGCTGATTGGTGATGGCGTTAT	781
EcCS	TTATGAAATGTGCAATTTGGCTTCAAGATCGTTGTCCAACTCTCTTCCAGGTCCATATGTATTTTCAA	852
EcCSopt	TTATGAAATGTGCAATTTGGCTTCTCGTCTCTGTCTAAATCTCTTCTGGTCCATATGTCTCTTTCAA	852
EcCS	GTGTGATTTGGCTAAAGCTGAGACCAACCATTGCCAGCAACACCATTGGAAAGCTGTGTGTATTA	923
EcCSopt	GTGTGATTTGGCTAAAGCTGTGCTGCAACCACTTGCCAGCAACACCATTGGAAAGCTGTGTGTATTA	923
EcCS	TACCAACCAATGAGGAAACCAATGAGTTGAATGAGTTAGCGGGAAGTTCAAGAAAGTTGAACG	994
EcCSopt	TACCAACCAATGAGGAAACCAATGAGTTGAATGAGTTAGCGGGAAGTTCAAGAAAGTTGAACG	994
EcCS	AATTCTGCAATCTGCGCGCAACTCTAGGCTCAACGAAGAACCAGATTTATCATCCAAGGTAGCCATAT	1065
EcCSopt	AATTCTGCAATCTGCGCGCAACTCTCTCTCAACGAAGAACCAGATTTATCATCCAAGGTAGCCATAT	1065
EcCS	TGTTGACCAATCTGTTCCCTTTCTATGACATAGATTTGGTTGGGGTAAACCTCTATGGGTAC	1136
EcCSopt	TGTTGACCAATCTGTTCCCTTTCTATGAAATGATTTGGTTGGGGTAAACCTCTATGGGTAC	1136
EcCS	TACGAACCTCTATGGTTCAAGATATTATTTGCTGCAAAAAACAAAGAGGTGATGGTATGAACCTT	1207
EcCSopt	TACGAACCTCTATGGTTCAAGATATTATTTGCTGCAAAAAACAAAGAGGTGATGGTATGAACCTT	1207
EcCS	GGGTCTCATGGAAGAAAAAGATGGCTCTTTCGAACAGCATGATATATTCATATGCTGCAAC	1278
EcCSopt	GGGTCTCATGGAAGAAAAAGATGGCTCTTTCGAACAGCATGATATATTCATATGCTGCAAC	1278
EcCS	AATCCATCTGTTCTTGCAGCATATTTCGCGATGTCA	1314
EcCSopt	AATCCATCTGTTCTTGCAGCATATTTCGCGATGTCA	1314

Supplemental Figure 3 | Nucleotide alignment of EcBAHD7 (cocaine synthase) and the codon optimized version of EcBAHD7 (EcBAHD7opt)



Supplemental Figure 4 | ALDI-SRM/MS images with pixels of 1x1 mm. Cocaine trace is shown in blue at m/z 304 > m/z 182, cinCOC trace is shown in pink at m/z 330 > m/z 182 and EME trace is shown in green at m/z 200 > m/z 182 in leaf stage 1, 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine- d_3 . The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine- d_3 : white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. *adaxial leaf surface; **abaxial leaf surface.



Supplemental Figure 5 | MALDI-SRM/MS images of non-treated and chloroform treated *E. coca* leaves after semi-automated spraying of the matrix. Cocaine trace is shown in blue at m/z 304 > m/z 182, cinnamoylcocaine trace is shown in pink at m/z 330 > m/z 182 and methylecgonine trace is shown in green at m/z 200 > m/z 182 in leaf stage 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine- d_3 . The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine- d_3 : white pixels give the highest signal; dark pixels give the lowest signal. Images are acquired with pixels size of 1x1 mm. *adaxial leaf surface; **abaxial leaf surface.

CHAPTER 4. Development and application of Liquid Extraction Surface Analysis – Differential ion Mobility Mass Spectrometry - *A new analytical platform for the analysis of drugs and metabolites directly in tissue sections*



As a complementary tool to MALDI, the 4th chapter introduces a new analytical platform based on liquid extraction surface analysis (LESA) and differential ion mobility spectrometry (DMS), hyphenated for the first time for the investigation of drugs of abuse in tissue sections, including structural isomers, after their separation in the gas-phase prior to their detection by mass spectrometry.

This chapter is divided in three parts:

Part 1 – Automated Liquid Extraction Surface Analysis, is a brief review of the principle and evolution of the surface sampling techniques based on a liquid extraction. This section will emphasize the liquid extraction surface analysis (LESA) mode available through an automated commercialized robot followed by chip-based nanoelectrospray ionization, used during the PhD work;

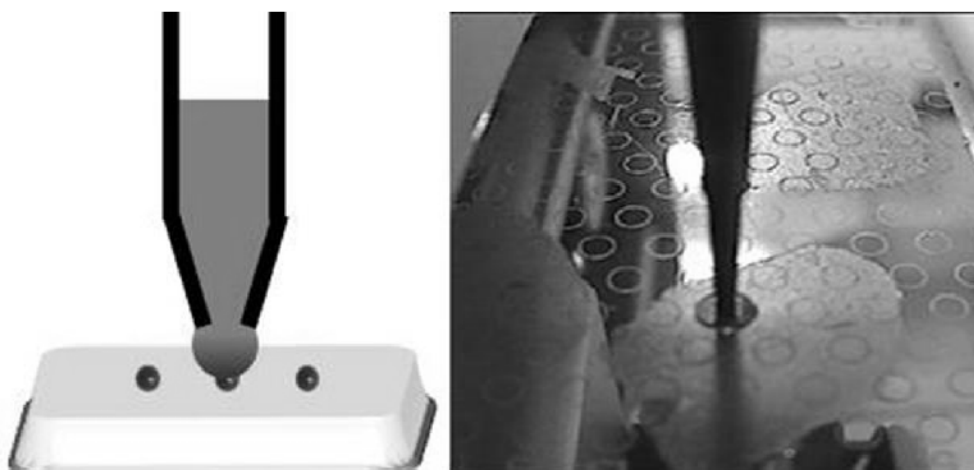
Part 2 – Ion Mobility Separation methods, presents the growing interest in ion mobility and the benefit provided by this technology in biosciences. This part reviews the two main operational principles used in ion mobility systems: i) the ion mobility spectrometry (IMS), which is a time-of-flight technique based on the measurement of the velocity of ions drifting under the effect of a low electric field (DC); ii) the differential ion mobility spectrometry (DMS) or field-asymmetry ion mobility spectrometry (FAIMS), developed on the basis of the measurement of the difference between ion mobility behavior in high and low electric fields.

Part 3 – Gas-phase separation of Drugs and Metabolites using Modifier-Assisted Differential Ion Mobility Spectrometry Hyphenated to Liquid Extraction Surface Analysis and Mass Spectrometry, presents an alternative generic approach to traditional LC-MS for the analysis of drugs of abuse. The platform integrates liquid extraction surface analysis for analytes tissue extraction followed by differential ion mobility spectrometry (DMS) for analytes gas-phase separation, including nominally isobaric analytes. This section characterizes the different parameters influencing the gas-phase separation in DMS (i.e. separation and compensation voltages, organic modifiers added in the drift gas). This work also assesses the quantitative performance of the LESA-DMS-MS platform (i.e. linearity, limits of detection and quantification, accuracy and precision, intra- and inter-sections variability).

CHAPTER 4.

Development and application of LESA-DMS-MS/MS

Part 1 – Automated Liquid Extraction Surface Analysis



4.1.1. Development of liquid extraction surface sampling probe/ionization

Over the last decade, atmospheric pressure surface sampling and ionization combinations prior to a mass spectrometric detection have been extensively explored, especially because of their molecular identification capabilities and ability to reach low detection levels. Among these techniques, liquid extraction surface sampling techniques are found particularly of interest as they can be followed either by electrospray or atmospheric pressure chemical ionization (ESI and APCI, respectively), can be (in theory) hyphenated to any types of analyzers, and allow selective extraction *via* the choice of the extractive solvent.

These surface sampling probes have been first developed as a read-out for planar chromatography (e.g. high-performance thin layer chromatography (HPTLC) or gel electrophoresis) on the basis of bringing a confined solvent stream to the surface of the sample, with the objective to dissolve the analytes present, aspirate/extract them and then carry through the detector. Two types of liquid extraction surface sampling probes are identified: the sealing surface sampling probe (S-SSP) and the liquid microjunction surface sampling probe (LMJ-SSP), of which principles, features and applications are presented in parts 4.1.1.1 and 4.1.1.2, respectively.

Part 4.1.2. is especially dedicated to the automated liquid extraction surface analysis (LESA) mode available on a robot equipped with a chip-based ionization source, which has been used during this PhD work for the investigation of drugs of abuse in tissue sections.

4.1.1.1. Sealing Surface Sampling Probe (S-SSP)

The sealing surface sampling probe (S-SSP) device was originally developed by Luftmann and coworkers for the *on-line* coupling of TLC with electrospray mass spectrometry.¹ To get better performance in term of precision and speed of operation, the device has been rapidly automated.²⁻³ The automated device consists of four main parts:

- 1) The stainless steel plunger, mounted on a linear actuator (z-axis), comprises a solvent inlet and an outlet capillary and is ended with a circular cutting edge (Figure IV- 1) designed to penetrate the substrate (e.g. initially the HPTLC plate);
- 2) A x,y-axis motor (liberty of motion of 20 cm x 20 cm) allows for analyzing large areas and thus large TLC plates, which is usually not possible with current commercialized apparatus, such as the ones fitted with MALDI sources, where only part of the plate can be analyzed and then requires plate segmentation and multiple analyses;
- 3) A camera acquires the image of the plate; its exact position is integrated into the software for selection of the region of interest to be sampled and analyzed;
- 4) An automated six-port valve enables passing from standby mode to extraction mode, and vice versa, in an automated way.

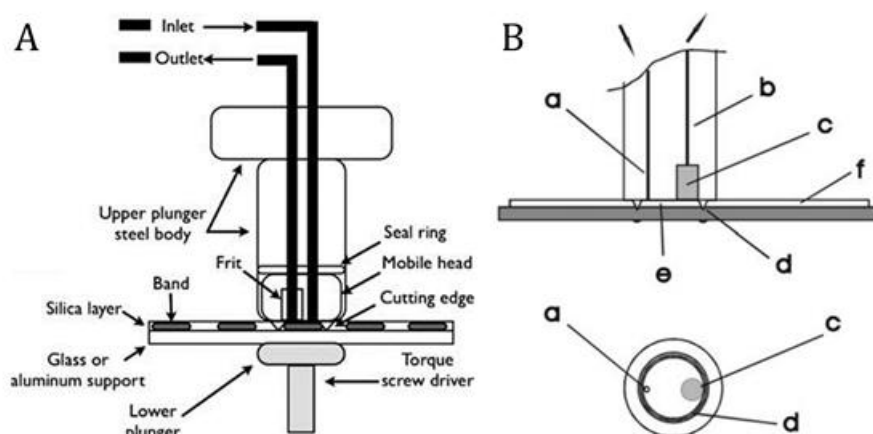


Figure IV- 1. Details of the stainless-steel plunger of the S-SSP. (A) full view and (B) cross-sections showing: (a) inlet capillary, (b) outlet capillary, (c) filter frit, (d) cutting edge, (e) adsorbent tablet and (f) TLC plate. Adapted from ref.¹⁻³

In standby mode, the solvent flow supplied by a conventional HPLC pump (at a usual flow rate of 0.1 mL/min) passes through the six-port valve to the mass analyzer. In extraction mode, after the plunger has been positioned in contact with the band of interest to form a tight seal by applying an appropriate pressure, the six-port valve is automatically switched to the extraction position (Figure IV- 2). The MS-compatible extractive solvent enters the inlet capillary of the plunger, dissolves the analyte and the solvent/analyte mixture is carried out to the mass analyzer. A 5- μ m frit protects the outlet capillary from clogging. Between two extractions, the plunger is automatically cleaned with pressurized air, but sample carryover remains possible and can be completely eliminated by extracting a blank spot on the plate.⁴ At these operating conditions (*i.e.* flow rate of 0.1 mL/min; plunger with 4 mm-diameter), the analysis time, which corresponds to the elution time of the compound from the sample plate to its detection, is estimated to be approximately 1 min.¹ The motor step resolution is of 6.5 μ m for the x,y-axis and of 1.5 μ m for the z-axis, which enables sampling of the surface of the sample with high precision. The probe has been designed with a diameter of the circular cutting edge of 2 mm or 4 mm, and has also been developed with an elliptical shape of 2 x 4 mm to better fit the TLC separated band size and shape, for more effective sampling.^{2,5}

As reviewed by Pasilis and Van Berkel,⁴ the field of applications of the S-SSP has been limited to the analysis of low molecular weight compounds, including: contaminants in food,⁶ pharmaceuticals in solid formulations,⁷ vitamins in energy drinks,⁸ lubricant additives in mineral oil,⁹ and metabolites,¹⁰ mainly from polar to moderately polar stationary phases. The application cannot be extended to non-polar (RP-8 and RP-18) stationary phases, because they cause leakage at the plunger-to-surface seal. The suitability of the sealed surface sampling interface has been assessed for quantitative analysis by HPTLC/MS down to low-ng/pg range per band, without the use of internal standard² and also validated by using a stable isotope (*i.e.* a deuterated analogue of the analyte of interest) to correct the

positioning of the plunger that is operated manually.³ The use of S-SSP has been extended to the analysis of targeted drugs and their metabolites in dried blood spots and mouse thin tissue sections.¹¹

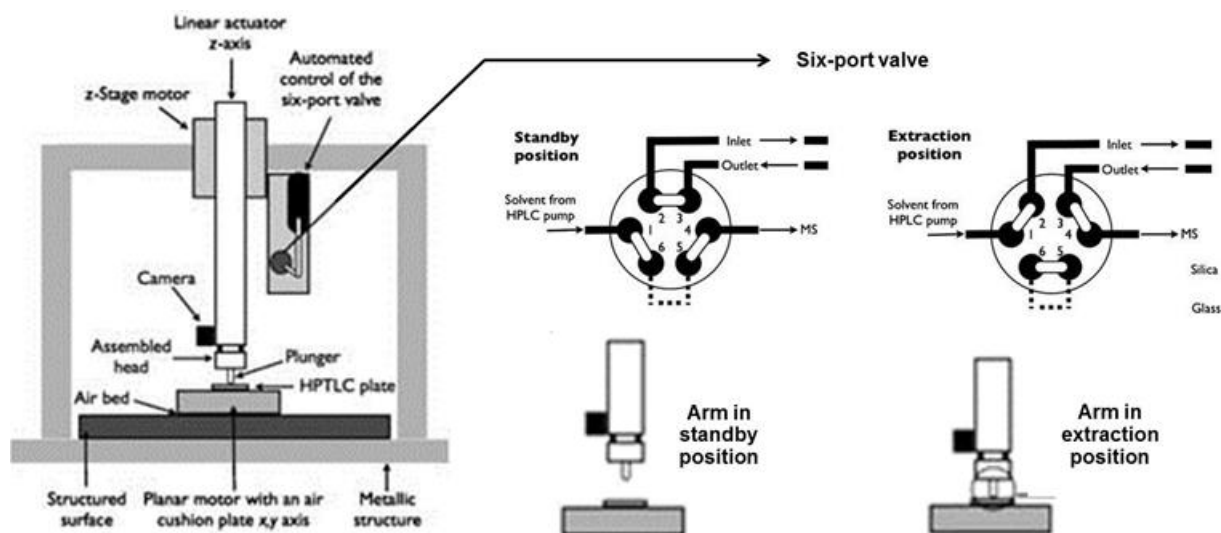


Figure IV- 2. Scheme of the automated S-SSP for hyphenation of planar chromatography with MS detection. Adapted from ref.²⁻³

4.1.1.2. Liquid Microjunction Surface Sampling Probe (LMJ-SSP)

The liquid microjunction surface sampling probe (LMJ-SSP) has been developed by Van Berkel and coworkers, and is mainly used in combination with MS subsequent to ESI¹²⁻¹⁶ or APCI sources.¹⁷

The LMJ-SSP device combines i) a surface sampling probe-to-surface liquid microjunction, of which thickness (*i.e. ca.* 300 – 500 μm ¹⁵) is controlled in an automated fashion throughout the duration of the extraction process, and ii) a self-aspirating ES emitter, built on the basis of the sampling probe / ES emitter first described by Wachs and Henion in 2001.¹⁸ The geometry of the LMJ-SSP interface consists of:

- 1) A stainless steel tee, where capillary tubes are arranged to provide a continuous flow of solvent, provided by a syringe pump, from the sampling probe end to the spray end (Figure IV- 3, inset A);
- 2) The sampling probe, which uses co-axial inner and outer capillary tubes (with an annular space in-between) to deliver the solvent to the surface sample (Figure IV- 3, inset B);
- 3) The self-aspirating ES emitter, located at the spray end and directed toward the entrance of the MS. The “self-aspiration” of the solvent, and thus of the dissolved analyte(s) from the surface of the sample, is promoted by a vacuum created locally by the combination of the adjustable pneumatic nebulization and the electrostatic field created by applying the high ES voltage to the tee. The solvent aspiration rate is

up to approximately 50 $\mu\text{L}/\text{min}$ in this configuration.¹⁴ The solvent/extracted analytes are then carried out and sprayed onto the mass analyzer;

- 4) An x,y,z-positioner, which holds the sampling probe and facilitates the optimization of the sprayer positioning toward the entrance of the mass analyzer. The control of the positioner is computer-assisted to allow automation of the surface sampling process;
- 5) A CCD camera, which is focused on the sampling end to facilitate the positioning of the surface relative to the sampling probe.

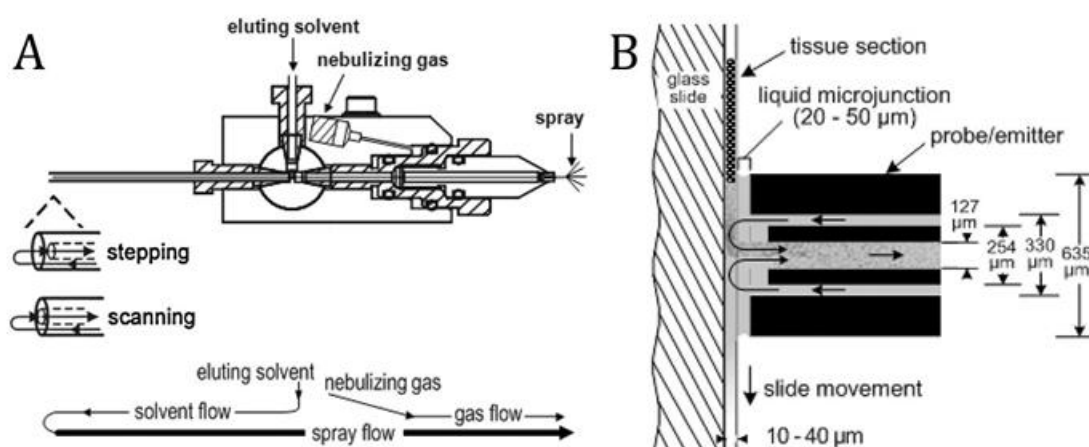


Figure IV- 3. Scheme of the liquid microjunction surface sampling probe (LMJ-SSP) for hyphenation with MS detection (from ref.¹²). A: details of the formation of the liquid microjunction in-between the sampling probe and the surface of the tissue section; B: detail of the sampling / emitter probe.

Three modes of action are available with the LMJ-SSP device: the stepping sampling mode, the scanning mode and the imaging mode.¹²

The stepping sampling mode¹² (also called *spot sampling mode* or *discrete mode*) consists of manually positioning the sampling probe over a particular position on the sample (e.g. TLC plate, tissue section) and a stable liquid microjunction is then formed between the sampling probe and the flat sample, usually spaced 100 μm from each other. Physical contact between the sample and the sampling probe is avoided as it may affect the solvent flow rate during the extraction process. Moreover, in stepping mode, the inner capillary (at the sampling probe end) must be kept at *ca.* 100 μm from the end of the outer capillary while the plate is positioned at a short distance back from the sampling probe and moving to a new sampling position (Figure IV- 3.B). This prevents the solvent from spraying out of the back of the capillary toward the sample. Considering the sampling probe outer diameter, the spatial resolution achieved in the stepping mode is of *ca.* 635 μm . This is particularly suitable for screening and profiling at a particular position on the TLC plate or on a tissue section at the scale of an organ. Such relatively high resolution is enough when the purpose of the study is not to image the distribution of a particular compound at the cell scale.

In the scanning mode,¹² the liquid microjunction formed in-between the sampling probe and the sample is $< 50\ \mu\text{m}$ thick to prevent a break and the sample holder is continuously moved relative to the probe. In opposition to the stepping mode, the inner capillary at the sampling end must fit to the outer capillary end when the device is operated in the scanning mode in order to keep stable the LMJ (Figure IV- 3, inset A). Applied to the analysis of a HPTLC plate, the scanning mode enables a complete automated read-out of a development lane showing separated analytes.^{12, 15} The construction of a 3D map for instance linking the relative abundance, the m/z and the time corresponding to the path of the probe during the development of a lane enables the analysis of more complex samples (compared to dies mixture) such as plant extract, and the identification of several compounds contained in a single spot after migration of the extract on the TLC plate (Figure IV- 4).¹³

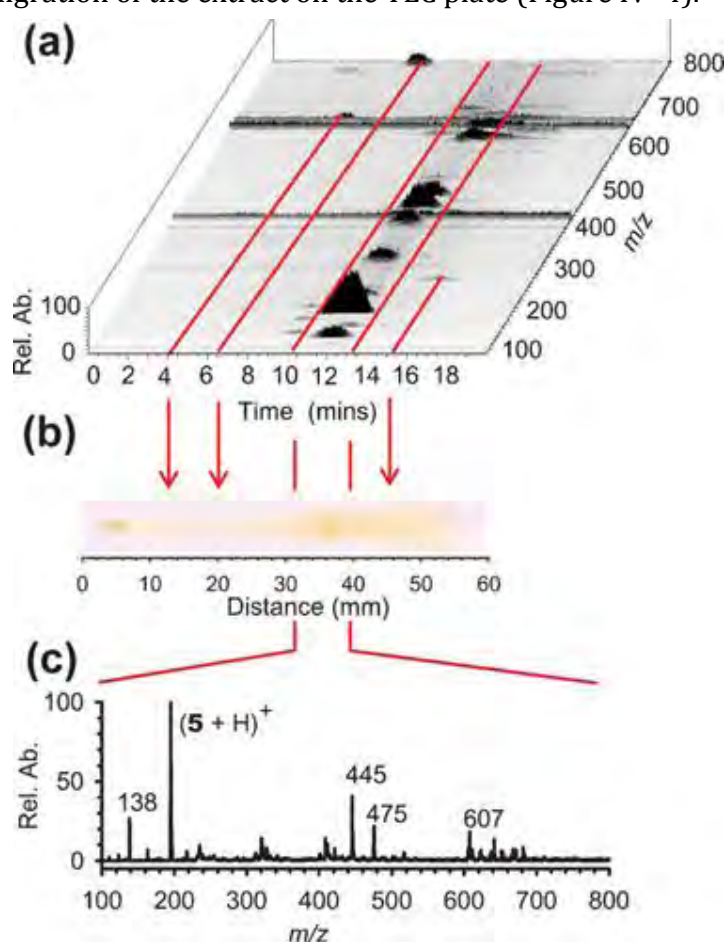


Figure IV- 4. TLC-ES-MS data collected from the separation and the LMJ-surface sampling analysis of a plant extract (*Ilex vomitaria*) showing: (a) the 3D map of relative abundance, m/z and time obtained by scanning the development lane shown in (b); photograph of iodine-stained development lane from the separation of the plant extract; (c) background-subtracted averaged mass spectrum. Reproduced from Ford et al., 2004.¹³

The optimization of the surface scan speed, the eluting solvent flow-rate and the read-out resolution has been investigated and shows that increasing the eluting solvent flow-rate to its maximum (up to $\sim 50\ \mu\text{L}/\text{min}$) allows surface scan rate up to $1\ \text{mm}/\text{s}$ without losing read-out resolution. However, these optimal conditions that tend to produce high

throughput analysis also hamper the quality of the data collected (e.g. in full MS scan mode or product ion scan mode for confirmation of the identity of the compound by collecting structural information) and the sensitivity achieved.¹⁴ The most likely hypothesis is that with the highest eluting solvent flow rate and with the fastest surface scan speed, the signal decreases significantly due to the dilution of the extracted material from the sample plate, but the ES ionization may also become less efficient. As a compromise, the use of more selective analysis modes such as the selected reaction monitoring mode, allows targeting of compounds at low concentration levels, equal to those achieved at lower throughput but with slower scanning modes (i.e. full MS and PIS modes).¹⁴

Finally, the imaging mode is based on the scanning mode and consists of scanning multiple lines over the sample. In this case, dedicated software programs are required to link the signals collected with their actual position by recording the time-MS signals on one hand (e.g. the SRM signal¹⁵), and the time-x-y-z data set on the other (from the computer controlling the x,y,z-stage), in order to generate a x-y-SRM ion current signal data set to visualize the results.¹⁵

As described previously through the examples shown to point out the interest and the modes of operation of the surface sampling strategy using the LMJ-SSP, the suitability of the device has been well established for studying dye mixtures from reversed-phase HPTLC plates at low-nanogram detection level of substance per spot (TLC),^{12-15, 19} at a relatively high throughput due to automation. Its complementarity to whole body autoradiography (WBA) has also been demonstrated lately for detecting drugs and metabolites in thin tissue sections without requiring any labeling¹⁶ and its application has been extended to the analysis of dried blood spots.

The effectiveness of the LMJ-SSP has also been demonstrated for quantitative purposes without internal standards.¹⁹⁻²⁰ Hydrophobic treatments have also been found favorable for the analysis of wettable surfaces.^{14, 21-22}

4.1.2. The Liquid Extraction Surface Analysis (LESA) mode available on an automated chip-based nanoelectrospray ionization robot

On the basis of the stepping mode available on the LMJ-SSP developed by Van Berkel et al.,²³ the LMJ-SSP has been implemented on a fully automated robot, the TriVersa NanoMate commercialized by Advion BioSciences (Ithaca, NY) and equipped with a chip-based nanoelectrospray ionization source.²⁴ The robot, previously capable of chip-based infusion and LC-MS fraction collection is now designed to perform direct surface analysis *via* the liquid extraction surface analysis (LESA) mode.

This section describes the different steps of the TriVersa NanoMate operated in the LESA mode, and introduces the principle of chip-based nanoelectrospray ionization (nES).

4.1.2.1. Automated liquid extraction surface analysis with the TriVersa NanoMate

The detailed steps involved in the LESA process are illustrated in Figure IV- 5. First of all, the arm of the robot picks-up a conductive pipette tip and moves to the solvent reservoir to aspirate the extractive solvent (Figure IV- 5.a,b). The pipette tip is then moved above the area to be sampled and dispenses a determined volume (usually 0.5-1 μL) on the top of the target. A liquid microjunction is formed in-between the pipette tip and the top surface of the sample (Figure IV- 5.c). This allows the static extraction of the analytes present within the tissue section (Figure IV- 5.d). The liquid extract is then aspirated back into the tip and infused through a nanospray nozzle of the silicon-based microchip (Figure IV- 5.e). The nES is then generated by applying a high voltage (i.e. 0-3 kV) to the conductive pipette tip and back pressure (i.e. 0.3-0.5 psi, usually using nitrogen as gas) to the liquid.

The choice of the extractive solvent composition is by far the most important parameter to be optimized. To extract low molecular weight compounds from tissue sections, the following criteria must be considered:

- high organic content improves the extraction efficiency (i.e. solvent typically used in routine methods to extract drugs from tissue homogenates),
- allows a stable liquid micro-junction in-between the pipette tip and the tissue section;
- stable nES spray;
- limits the dispersion across the surface.

During the method development carried out during this PhD work, methanol has been mainly used at a proportion of 70-80%. With higher organic content up to 80%, the liquid micro-junction between the pipette tip and the tissue section was still stable but the nES became unstable. Although MeOH is a good solvent to extract small drugs, it also limits the dispersion across the surface compared to other organic solvents such as IPA or ACN.²⁵

One could imagine that the main limitations of the process would be the clogging of the nozzles by particle extraction from biological material such as tissue sections. Fortunately, to our knowledge and the literature, this phenomenon was never observed.²⁴

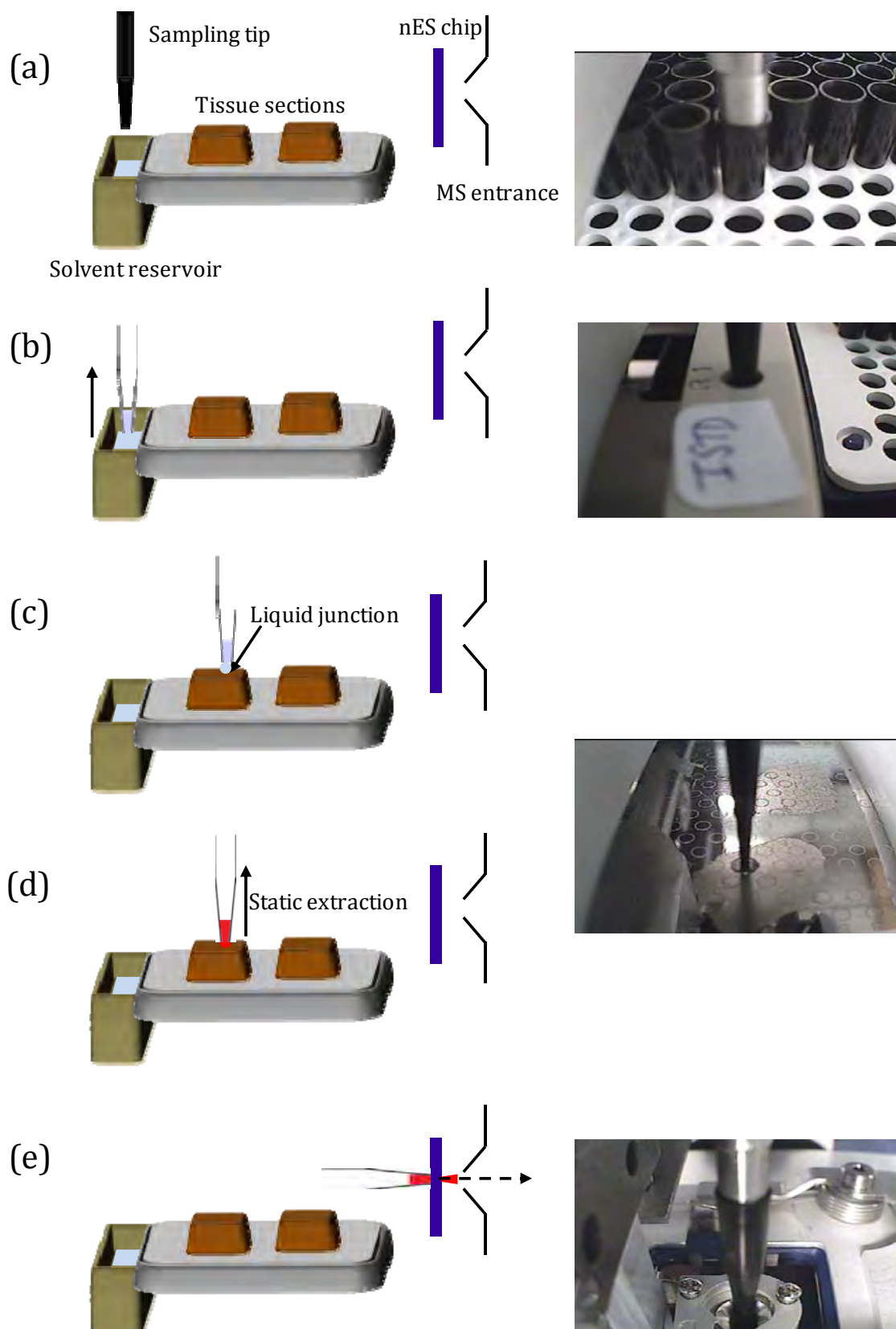


Figure IV- 5. Mode of operation of the fully automated liquid extraction-based surface sampling using a chip-based robotic nanoelectrospray platform and corresponding pictures. (a) Pipette tip picked up; the solvent is (b) aspirated and (c) dispensed; (d) analyte static extraction; (e) spray sample and acquisition of the signal by the mass analyzer. Adapted from Kertesz and Van Berkel, 2010.²⁴

4.1.2.2. Chip-based nanelectrospray ionization

The TriVersa NanoMate system serves as the nanelectrospray ionization (nanoESI) source and can be mounted on any type of mass analyzer thanks to a customized mounting bracket. The robot holds a single ESI Chip containing a 20 by 20 array of micro-fabricated nozzles, etched to a depth of 50 μm in a silicon wafer (Figure IV- 6.a). The 400 nozzles contained in one chip are identical, which provides very reproducible electrospray results.²⁶ The inert coating on the surface allows a variety of acidic and organic compositions and concentrations to be used in order to promote ionization without degradation of the nozzles.²⁷

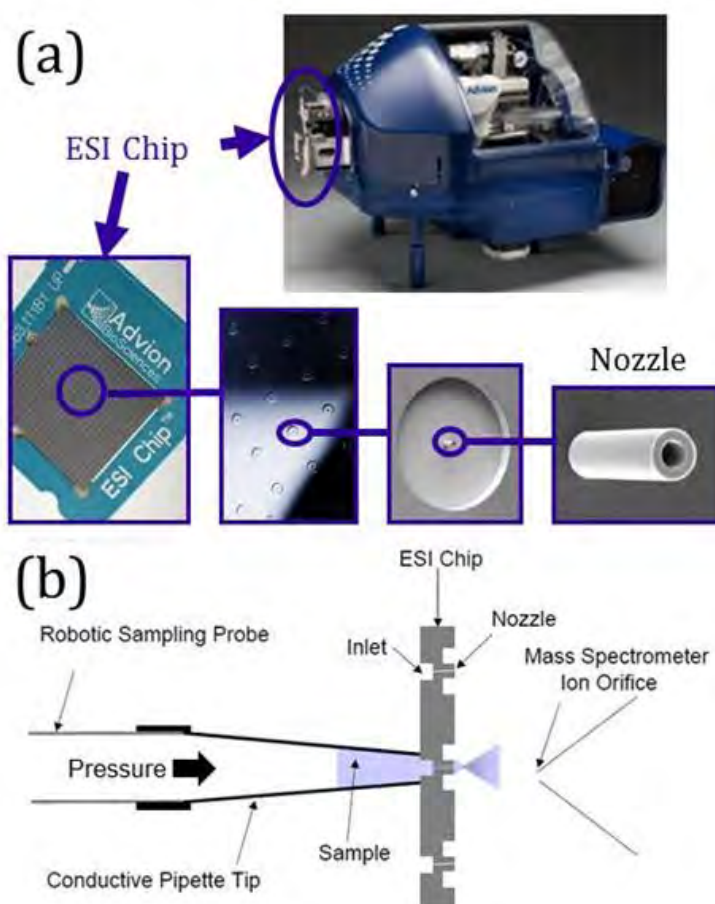


Figure IV- 6. Pictures of the TriVersa NanoMate robot and a silicon-based nESI chip. (a) One chip contains 20 x 20 nozzles and allows for 400 analyses; (b) generation of the nES by applying a high voltage to the conductive pipette tip and a pressure to the liquid sample. Each nozzle and tip is single use, which avoid sample-to-sample carry-over or autosampler contamination. Adapted from Advion.com

The generation of the nES is achieved by applying a high voltage and a pressure to the sample contained in a conductive pipette tip. The internal diameter of the nozzles varies from 2.1 to 5.5 μm , designed to generate nESI from liquid samples at flow rates from 20 to 500 nL/min. In conventional electrospray devices, the electric field is defined by the potential difference between the spray capillary and the mass spectrometer inlet. nESI chip integrates a grounded silicon electrode and the electric field is formed from the potential

difference between the conductive silicon substrate and the voltage applied to the fluid via the conductive pipette tip. Because the distance is fixed and only a few microns, the high electric field formed is uniform around the nozzle tip and denser compared to the one generated by a conventional pulled capillary (Figure IV- 7). The distance that defines the electric field is *ca.* 1'000 X smaller than the distance of the nozzle to the mass spectrometer. In this configuration, the ESI process is decoupled from the inlet of the mass spectrometer. It results that the MS position and voltage no longer play a significant role in forming the electrospray. With only a few μL of sample, the unique field strength created by the nES nozzles allows for a spray over several minutes, more efficient and more stable than the spray generated in conventional ES devices.²⁶

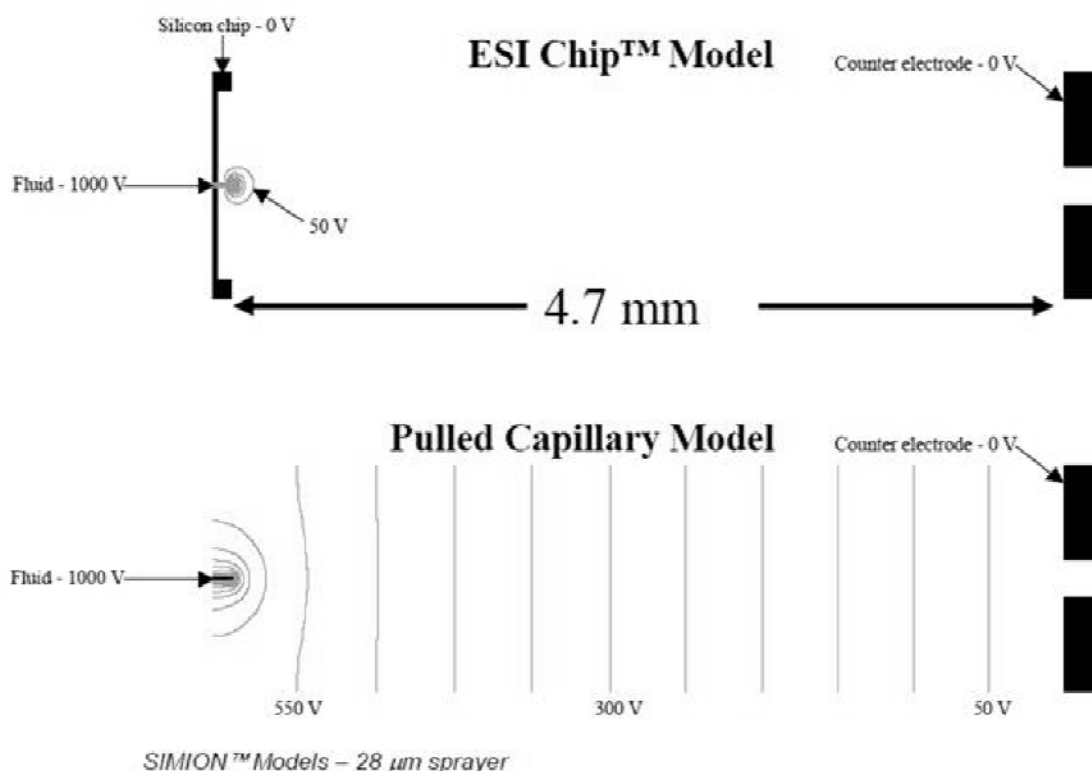


Figure IV- 7. Comparison of the electric field generated by a ESI chip (upper scheme) to the conventional pulled capillary model (lower scheme). From Advion.com

4.1.2.3. Dedicated software for fully automation of the LESA process

A ChipSoft Manager software (v.8.3.2.1209, Advion BioSciences) is used to control automatic liquid extraction from a surface sample via the TriVersa NanoMate. Several parameters can also be optimized, like the distance between the tip and the sample, the aspirated and dispensed volumes, the spray pressure, the spray voltage. In addition, automation is facilitated by the implementation of a dedicated driver ("Advion driver") provided by Advion BioSciences. Thus, a picture of the sample is taken (by a scanner) and imported into the driver. The selection of discrete spots to be sampled is directly made from this picture and a batch is generated. The batch is then sent to the Analyst software (v. 1.5, AB Sciex), which is used for controlling the mass spectrometer and collecting data.

4.1.2.4. Applications

Although often criticized by the MS imaging community for the poor spatial resolution achievable (i.e. 1.2-2.0 mm with 1 μ L of solvent deposited on the top of the surface), liquid extraction surface analysis has to be considered as an excellent tool for conducting profiling experiments from a selected spot. This allows for quick metabolite screening or parent drug localization to an organ.²⁰ Complementary to QWBA that provides distribution information on radiolabelled material, LESA followed by MS detection enables differentiation of a parent drug from its metabolites.²⁸

LESA has been successfully applied for the analysis of a large variety of analytes from any type of surface: dried blood spot;²⁴ whole body or organ thin sections from drug dosed mice/rat;^{20, 25, 28-29} food analysis;³⁰ microbiology³¹; solid-phase extraction cards.³²

4.1.3. Perspectives for future developments

With the objective to continuously improve the liquid extraction surface analysis approaches, several strategies have been envisaged and demonstrated as relevant for improving the desorption efficiency, the spatial resolution and for use in combination with orthogonal techniques to allow separation of isobaric species.

4.1.3.1. Improvement of the sample transfer efficiency

Recently, the combination of a laser ablation (LA) with electrospray ionization has proven a high efficiency of analyte transport and ionization (i.e. development of LAESI source,³³⁻³⁴ see chapter 2). As a consequence, the implementation of LA in the *conventional* liquid phase collection surface sampling workflow seems to be a logical evolution of the approach.

The group of Van Berkel reported very promising results with the LA-LMJ-SS approach over the last 2 years.³⁵⁻³⁸ The strategy consists of two main workflows:

- (a) direct transfer of the ablated material into a formed liquid microjunction^{35, 39} after adapting existing probes to produce a liquid droplet at the end of the tip, which is in close proximity (few hundred μ m) to the surface to collect the sample plume produced by laser ablation. This approach demonstrates a superior sampling resolution down to 10 μ m demonstrated (1-2 μ m should be possible).³⁶ LA can be combined either with continuous flow LMJ-SSP or LESA, to perform dynamic or static extraction, respectively (Figure IV- 8.a);
- (b) spatially resolved LA sample transfer followed by liquid extraction (Figure IV- 8.b);⁴⁰ this approach consists of transferring the biological material sampled in 10 μ m increments on a secondary surface with high spatial resolution (i.e. an array of points separated by 4 mm from each other) for subsequent read-out using LMJ-SSP in line scanning and spot sampling mode and LESA.

These strategies improve not only the sample transfer to the probe containing the extractive solvent, but also the spatial resolution of sample smaller areas (i.e. 10 to 200 μ m). Although

this phenomenon is not observed in conventional LMJ-SSP or LESA, one could expect the clogging of the nozzles by particles extracted from biological material, but nothing has been yet reported on this potential issue.

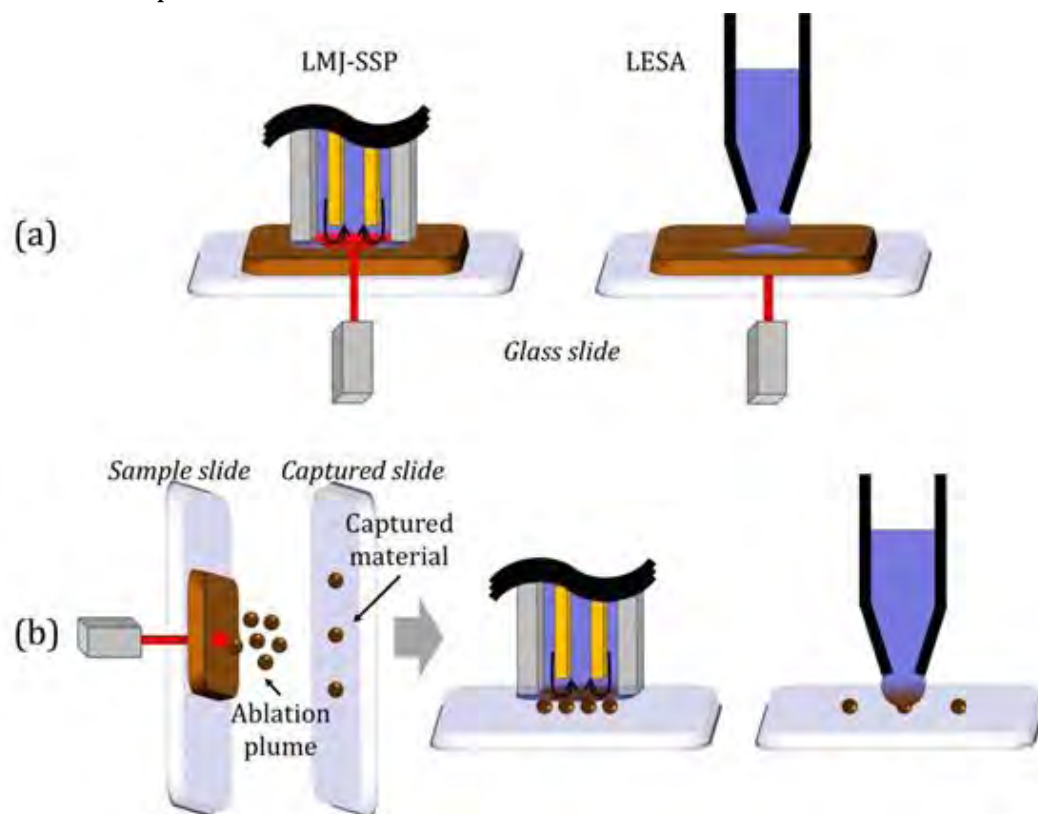


Figure IV- 8. Combination between laser ablation (LA) and liquid microjunction (LMJ) surface analysis. (a) LA directly into a formed LMJ; either with a LMJ-surface sampling probe (SSP) to perform dynamic extraction or LESA to perform static extraction. (b) Spatially resolved LA sample transfer followed by liquid extraction. Adapted from reference³⁶

4.1.3.2. Improving the spatial resolution by direct sampling with the contact closure strategy

Decreasing the area sampled is of utmost importance for the analysis of whole body mouse/rat or organs containing small features such as the brain, because it limits the micro-delocalization of the analytes within the liquid microjunction created on the top of the tissue section.

For that purpose, LESA with contact extraction has been proven to improve spatial resolution. In this configuration, the pipette tip is plunged within the tissue section. Based on the principle that the pipette tip has an internal diameter of 400 μm , the sampling diameter is therefore of 400 μm (verified by microscopy). This strategy also offers the advantage of limiting the dispersion of the extractive solvent in contact with the tissue section. This approach has been successfully applied in profiling spatial lipid species distributions in mouse brain.⁴¹ However, the authors do not report whether solid material is also extracted from the tissue section, which may cause the clogging of the nozzles.

Another strategy consists of modifying the conventional TriVersa NanoMate by using a fused silica probe instead of pipette tips. With a 170 μm OD-probe, the analytes are extracted from an area of 200-400 μm , which is lower compared to the 1.5-2 mm resolution achieved on standard LESA using pipette tips.⁴²

4.1.3.3. Adding a separation dimension after the liquid extraction

As often specified in this manuscript, one of the limitations of direct surface sampling is the absence of chromatographic separation beforehand which helps to resolve isomeric compounds or differentiate the analytes with isobaric endogenous compounds, when the performance of the following MS analyzer are not sufficient.

Because the surface sampling and the ionization processes are resolved both in space and time dimensions, one advantage of the liquid extraction surface sampling is the possibility of manipulating the analyte in the liquid phase prior to their ionization.²⁰ As a consequence, LESA can easily incorporate a liquid based separation after the surface sampling process, which is not possible with MALDI or DESI ionization techniques, for instance. Abu-Rabie and Spooner have demonstrated the suitability of LC-separation subsequent to sealing surface sampling probe (S-SSP) to eliminate matrix interferences for the analysis of drugs in dried blood spots on paper.⁴³ Kertesz *et al.* also developed a LMJ-SS approach followed by HPLC-ESI-MS for the analysis of drugs and metabolites in whole-body thin tissue sections, which helps to distinguish isomeric phase II metabolites of propranolol.⁴⁴ Continuous-flow LMJ-SS coupled on-line with HPLC/MS also enables the extraction, separation, and detection of proteins and low molecular weight compounds (e.g. drugs of abuse) from tissue sections and dried blood spots. Usually, dried blood spot surface is sufficiently hydrophobic for maintaining a stable liquid junction, even with high aqueous content solvents.⁴⁵

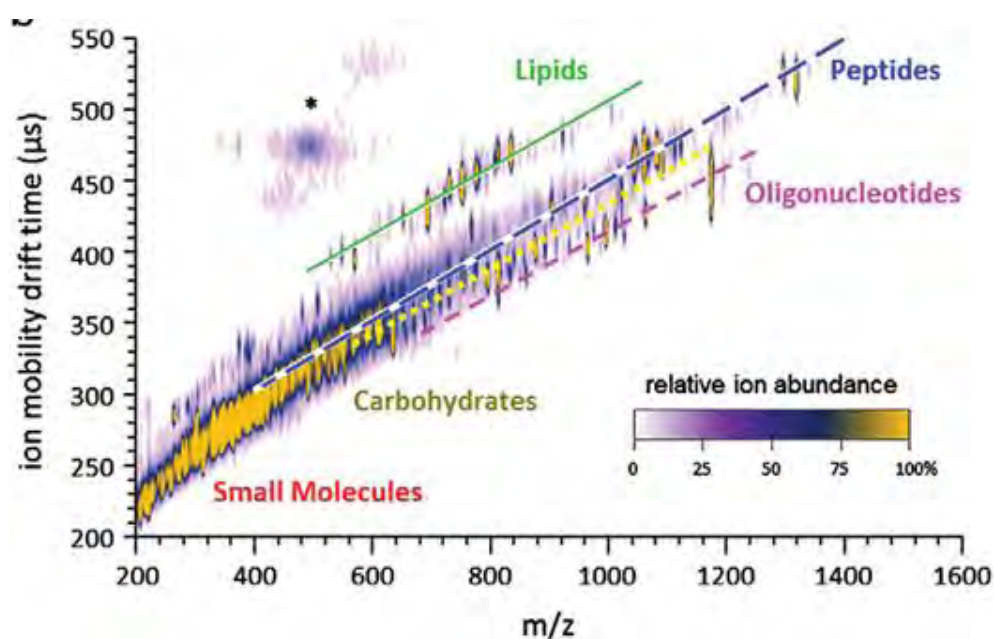
Finally, LESA-LC combines liquid extraction surface analyses with nanoLC separation, after the modification of the TriVersa Nanomate using a nano-port switch valve for automated LESA-LC operation.⁴² This new configuration provides an additional separation which helps to distinguish lipid isomers from a 200 μm diameter sampling area that would have overlapped in other imaging techniques like MALDI.

Recently, ion mobility has gained interest as a gas-phase separation method after direct surface analysis in place of separation based on liquid chromatography. Because the applications are not limited to atmospheric pressure liquid microjunction surface sampling/ionization techniques (i.e. also after MALDI or DESI), the growing interest in ion mobility and the benefit provided after direct surface analysis are presented in Part 2 of this fourth chapter.

CHAPTER 4.

Development and application of LESA-DMS-MS/MS

Part 2 - Ion Mobility Separation Techniques and benefits for mass spectrometry imaging



4.2.1. The growing interest in ion mobility separation in mass spectrometry imaging

Because biological materials such as cells, tissues or body fluids are complex, it requires a combination of multiple analytical methods to gain a fundamental insight into their chemical and biological information content. In addition, there are frequent cases where compounds are co-desorbed and the mass resolving power is not sufficient to resolve these compounds (e.g. isobaric compounds and interfering isotopic clusters). Multi-dimensional strategies are thus employed and typically, liquid chromatography or gas chromatography with mass spectrometric detection are the techniques of choice, after homogenization of the sample, with analysis times ranging from minutes to hours.

In chromatographic-free strategies (i.e. infusion, flow injection analysis, direct surface analysis for MSI), adding a new dimension is required to provide sufficient selectivity in order to resolve compounds with identical nominal mass but different elemental composition. This is especially true when analyzing complex mixtures or biological samples. This selectivity is typically provided by high resolution mass spectrometry or tandem mass spectrometry (MS/MS).^{29, 31} However, to deal with structural isomers or isobaric compounds, chromatographic-free approaches often suffer from the absence of separation beforehand, and high resolution is not adequate to distinguish the isomers.

Although the principle has been known a long time, ion mobility spectrometry (IMS) has been recently introduced as an alternate approach in combination with mass spectrometry imaging (MSI) and has proven a great benefit for its capability of resolving ions in the gas-phase on the basis of their collision cross-section (i.e. their physical size/shape).⁴⁶ The combination IMS-MSI therefore allows characterizing a molecule in its biological context based on its size, structure (molecular weight) and spatial distribution within the sample.

The following parts review the principles of the *conventional* ion mobility spectrometry, referred as drift-time ion mobility spectrometry (DT-IMS); the recently introduced travelling-wave ion mobility (commercialized by Waters; in combination with MALDI for MS imaging applications); and finally the differential ion mobility spectrometry (DMS) also called high-field asymmetric ion mobility spectrometry (FAIMS).

4.2.2. Principle of the conventional ion mobility – drift-time ion mobility spectrometry (DT-IMS)

4.2.2.1. Principle of DT-IMS

Ion mobility refers to the motion of free (gas-phase) ions in the presence of gas collisions within a pressurized chamber, usually held between 1 (reduced-pressure) and 760 Torr (ambient-pressure).⁴⁷ A conventional drift-time ion mobility spectrometry (DT-IMS) cell consists of a series of stacked-ring electrodes and uses lengthwise low electric fields (< 10 kV/cm) (Figure IV- 9). This technique is called “linear” because the ions travel at a constant

velocity through a linearly decreasing field.⁴⁸ In DT-IMS, ions are injected in packets into the ion mobility cell, and their *drift-time* is measured, when they are drifting through the cell and colliding with a collision gas (usually nitrogen, helium or argon). The drift-time is directly affected by the collision cross section of each gas-phase ion that can be identified and separated by their mobility K , directly proportional to the ion velocity (v_d) and the potential drop of the applied electric field (E) according to Equation 1:⁴⁹

$$K = \frac{v_d}{E} \quad \text{Equation 1}$$

K is strongly dependent on the physical properties of the ion, i.e. size, conformation, charge state of the ions.⁵⁰ Note that K is also strongly linked to the gas density, and is proportional to the ratio of the electric field strength over the number density of drift gas (E/N). In the literature, K is often defined in Td, which stands for Townsend ($1 \text{ Td} = 10^{-17} \text{ V.cm}^2$), the unit of E/N .⁵¹

The ion mobility depends on the ion-neutral interaction averaged over a number of collisions. If the thermal energy gained by the ion from the electric field is negligibly small in comparison with the thermal energy transferred due to ion-neutral interaction, ion mobility is independent of the field. As a result, the average interaction energy is equal to the thermal energy and the mobility coefficient is constant.⁵² Higher electric fields lead to the transfer of energy to the ions and change the ion-neutral interaction, and the ion mobility becomes therefore field dependent.

At low electric field condition, the ion-gas diffusion creates high ion losses, which explains why the DT-IMS method inherently suffers from low sensitivity.⁴⁷ Knowing the experimental parameters, the collision cross section can be directly calculated from Equation 2:⁴⁹

$$K = \left(\frac{3q}{16N} \right) \left(\frac{2}{kT} \right)^{1/2} \left(\frac{m+M}{mM} \right)^{1/2} \left(\frac{1}{\Omega} \right) \quad \text{Equation 2}$$

with: K : ion mobility constant (directly proportional to the electric field and the velocity of the ions)

q : charge on the ion

N : number density of the buffer gas

$$\begin{aligned} k: \text{ Boltzmann's constant} &= \frac{\text{gas constant}}{\text{Avogadro constant}} = \frac{R}{N_A} = \frac{8.314472 \text{ J.mol}^{-1}.\text{K}^{-1}}{6.022 \times 10^{23} \text{ mol}^{-1}} \\ &= 1.381 \times 10^{-23} \text{ J.K}^{-1} \end{aligned}$$

T : absolute temperature

m : mass of the buffer gas

M : mass of the ion

Ω : collision cross-section of the ion

The ion mobility drift-time plotted against the molecular weight of each ion follows a linear regression, which makes the ion mobility predictable for an analyte within a given class of compounds. As a result, compounds from different molecular classes such as lipids, carbohydrates, oligonucleotides or peptides can easily be isolated from each other.⁵³⁻⁵⁴

Although DT-IMS at ambient pressure can be used as a standalone spectrometer (e.g. detection of explosive or dangerous chemicals at airports⁵⁵⁻⁶⁰), it can be easily used in combination with other mass analyzers such as time-of-flight (TOF) instruments. However, although the DT-IMS operated at ambient pressure or with a gas buffer pressure of hundreds of Torr⁶¹⁻⁶² provide better resolution and better separation selectivity compared to the system operated at reduced pressure (i.e. < 10 Torr⁶³), it often appears that the transfer of the ions from the ion mobility chamber to the mass analyzer is inefficient, which considerably decrease the performances of the system in terms of sensitivity.⁴⁶ This is especially true when they are coupled to ion sources producing ions continuously. To overcome this issue, a cell can be implemented before the drift tube, to trap the ions and release them preferably in narrow ion packets to get better separation performance. But because of this accumulation of ions in front, these instruments have the disadvantage of a low duty cycle.

4.2.2.2. Interest for IMS in MS imaging

One of the greatest advantages of IMS is its ability to differentiate analytes that are isobaric but with different structures, from small pharmaceuticals⁶⁴, carbohydrates⁶⁵ or lipids^{47, 54, 66-67} to peptides and MDa protein complexes.⁶⁸⁻⁶⁹ In particular, IMS has been used in combination with MALDI ionization for imaging of biological surfaces.⁷⁰ The main articles report experiments performed on the SYNAPT G2 HDMS commercialized by Waters, which uses travelling-wave ion mobility, and are detailed in the following section “4.2.3. Travelling-wave ion mobility (TWIM).”

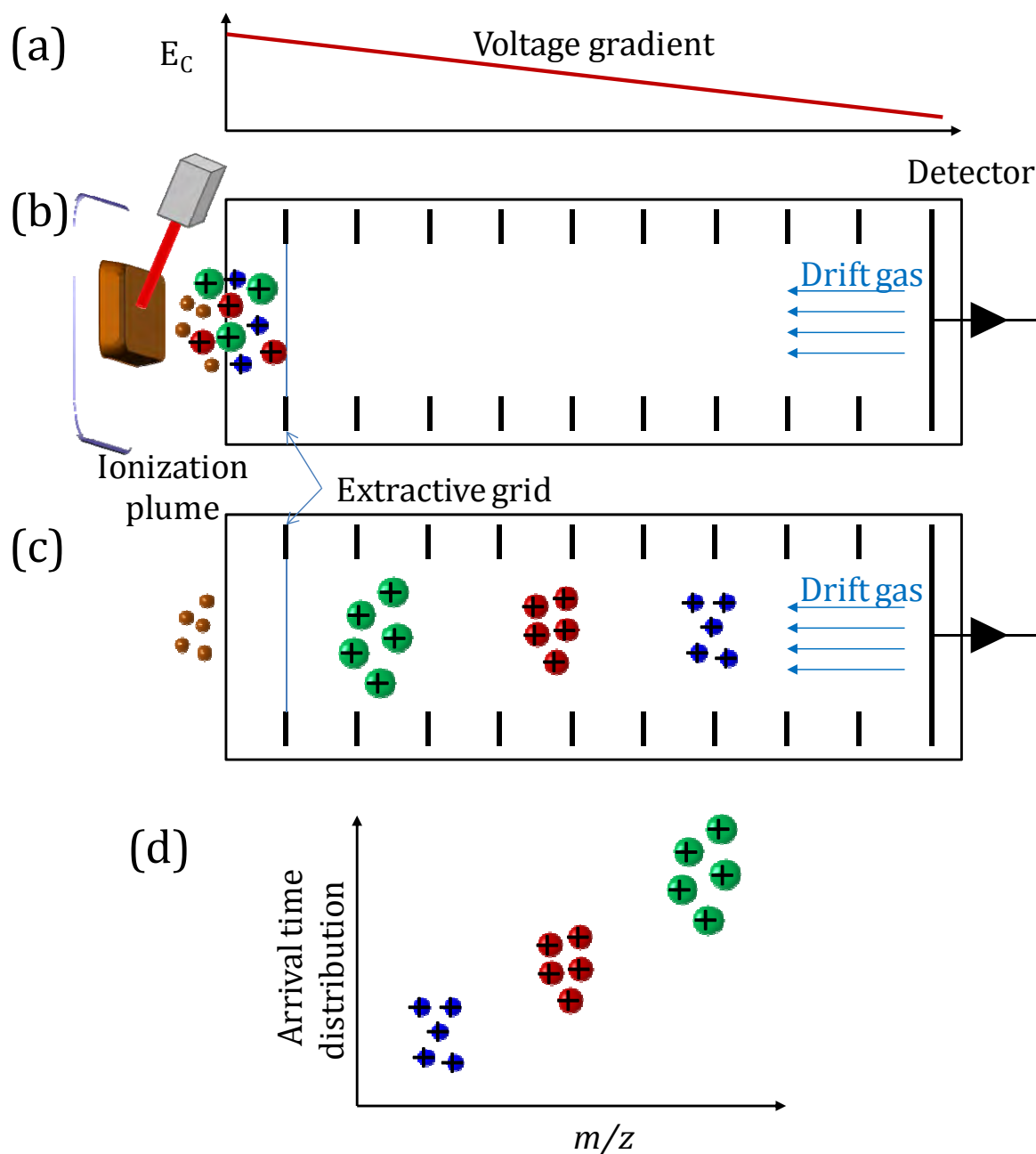


Figure IV- 9. Principle of drift-time ion mobility spectrometry. (a) linearly decreasing electric voltage applied through the drift tube; (b) ionization of the sample by MALDI; (c) while the neutral molecules (brown) do not enter the drift region, the ions are extracted and enter the drift region where they flow against the drift gas to reach the detector; (d) the drift plot shows the arrival time to the detector distribution in function of the size and shape (i.e. collision cross section) of the ions. Adapted and completed from O'Donnell *et al.*⁴⁸

4.2.3. Travelling-wave ion mobility (TWIM)

4.2.3.1. Principle of travelling-wave ion mobility (TWIM)

Travelling-wave ion mobility (TWIM) is the latest ion mobility technique developed and is very similar to DT-IMS because it is operated under low electric field conditions, but TWIM uses a migrating low voltage pulse to push ions through the drift gas.^{47, 71} This technology

was first described by Kirchner in 1993,⁷² later resumed by Giles *et al.* in 2004,⁷³ and finally commercialized by Waters Corporation under the trend name “T-Wave”. The information related to the technology are taken from Waters Corporation website.⁷⁴

On the same basis as conventional DT-IMS instrumentation, the ions are injected within an ion guide based on a stack of ring electrodes. The difference is that these electrodes can be supplied with radio frequency (RF) and direct current (DC) voltages, causing a non-uniform, moving electric field / voltage pulse to push ions through a neutral buffer gas. T-Wave IMS utilizes an opposite phase of RF applied to consecutive ring electrodes to radially confine the ions within the device, without disturbing their path along the axis (Figure IV- 10.a⁷³). This has the effect of slowing down the ions or even to produce a potential barrier that cannot be crossed by the ions. Additional to the RF voltage, a DC voltage can be applied successively over adjacent electrodes. As a result, the ion barrier moves forward, causing any ions in front of it to be propelled forwards. Stepping the ion barrier sequentially along the electrodes of the device to the other creates a travelling wave that drives the ions through the IMS cell (Figure IV- 10.b).

Waters Corporation commercializes the Triwave technology SYNAPT MS series instrument HDMS, equipped with either ESI or MALDI sources. The Triwave consists of three consecutive T-wave ion guides.⁷⁵ When experiencing the three consecutive T-wave regions, the ions are first trapped and accumulated to ensure no loss in sensitivity. Then, the ions are ejected in packets and driven into the second T-wave guide filled with a collision gas which slows down the ions, causing ions of different size, shape, charge and mass to transit at different rates and allow their separation. The separated ions are then transferred to the detector.

In addition, the T-wave can be used for collision-induced-dissociation (CID) fragmentation, either in the trap cell or after the mobility separation in the third T-wave guide (as a result of the second option, the fragment ions have the same drift time as the corresponding ion). MS³-like experiments can also be performed by fragmenting the ions in both cells.^{46, 76}

However, although the collision-cross sections of ions are accurately predictable, this is not the case with TWIM because the travelling wave voltages applied are difficult to characterize quantitatively and accurately.⁷¹

4.2.3.2. Benefits of travelling-wave ion mobility (TWIM) after direct surface sampling

As mentioned above, the SYNAPT (G2) HDMS can be either equipped with a MALDI source or an ESI source. As a result, a large panel of applications is possible as well as combinations with numerous ionization sources, both under vacuum condition (i.e. MALDI) or at ambient pressure conditions (i.e. DESI, LESA). The advances in MALDI imaging mass spectrometry-adding a new dimension of separation for direct tissue analysis has proven a real benefit to remove endogenous interfering ions.⁷⁷⁻⁸⁰ McLean *et al.* demonstrates the advantages of combining two-dimensional separation at each spatial location on the basis of ion mobility

prior to MS analysis for profiling of human glioma and selective lipid imaging from rat brain.⁵³ The MALDI-IMS-MS and MS/MS platform has also been used in the early-stage development study of vinblastine, a drug used to treat certain kinds of cancer. This work clearly demonstrates the benefit of ion mobility to remove interfering endogenous isobaric lipid ions, resulting in higher confidence in the imaging data, either after MS or MS/MS analysis.⁸¹ Applications in lipidomics following MALDI ionization have also been reported several times.^{54, 82}

Several studies have also reported the benefit of the approach to reveal new spatial distributions of tryptic peptide distributions on tissue surfaces,⁸³⁻⁸⁴ increasing the amino acid sequence coverage,⁸⁵ improving the peptide yield and protein identification for in situ proteomic investigation of frozen and formalin-fixed paraffin-embedded adenocarcinoma tissue sections⁸⁶ and characterizing interactions such as peptide-peptide, peptide-drug or drug-lipid.⁸⁷

Recently, the SYNAPT G2 HDMS capable of ion mobility separation has been used in combination with a liquid extraction surface analysis (LESA) to provide additional confirmatory evidence of detection in dosed tissue to differentiate between endogenous compounds or chemical noise. Again, it results from the conclusion that the IMS provides cleaner mass spectra by increasing the selectivity for the analyte of interest.²⁰

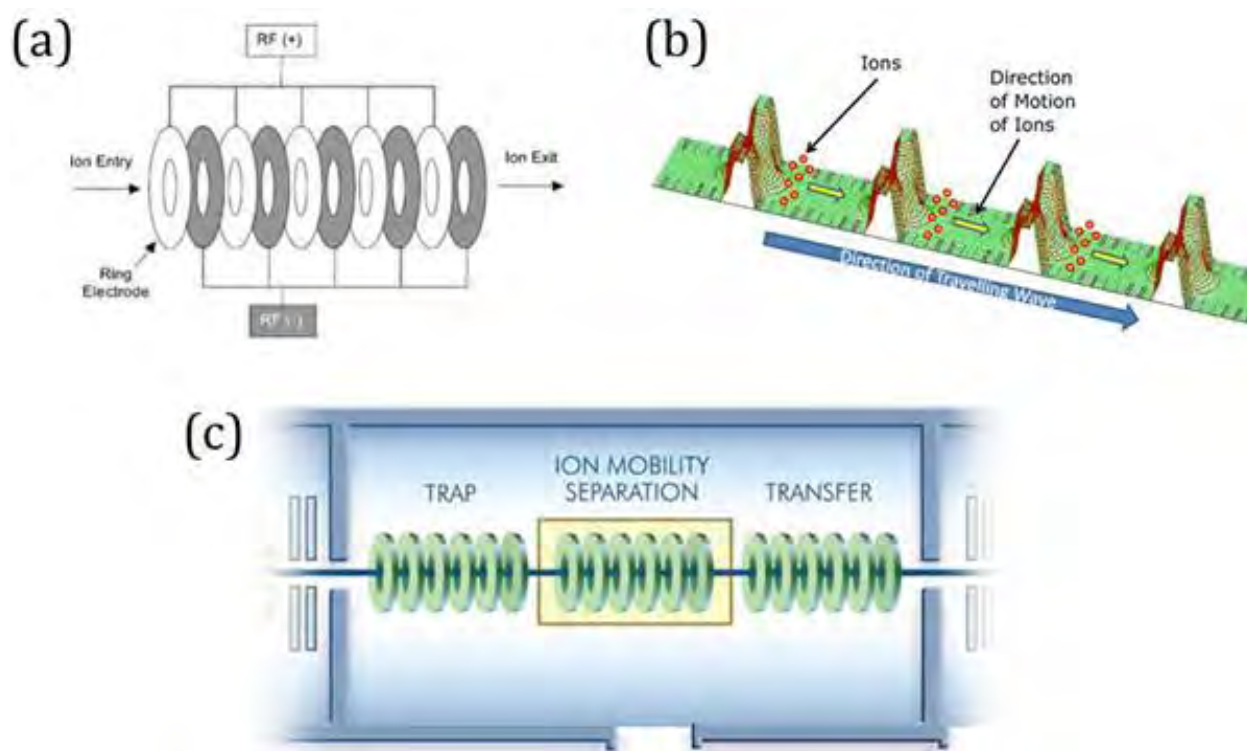


Figure IV- 10. Travelling-wave ion mobility. (a) scheme of an RF-only stacked ring ion guide; (b) ion motion following the travelling wave; (c) Three T-wave ion guides implemented on the SYNAPT MS series instrument HDMS. Pictures taken from (a) Giles et al.⁷³ and (b-c) Waters Corporation website⁷⁴⁻⁷⁵

4.2.4. Fundamentals of Differential ion Mobility Spectrometry (DMS) / Field-Asymmetry IMS (FAIMS)

4.2.4.1. Some definitions about “differential mobility”

To avoid any confusion, this paragraph begins with a short definition of the concept of differential ion mobility that is described and employed in this manuscript.

In the literature, we find “differential ion mobility” refers to two ion mobility separation techniques, which are first “asymmetric field ion mobility”, in reference to both DMS (which stands for differential ion mobility spectrometry) and FAIMS (which stands for field-asymmetric ion mobility spectrometry), and finally “differential ion mobility analysis”, commonly referred as DMA.

Contrary to DMS or FAIMS that use asymmetric fields, DMA uses uniform electric fields in-between 2 electrodes, one held at high voltage and the other one grounded. In such conditions, the uniform electric fields can be precisely controlled. When the charged particles experience the electrical field while simultaneously colliding with an inert background gas, they can be differentiated on the basis of their size. Because DMA operates in the gas-phase under elevated pressure conditions, DMA is usually used after an electrospray ionization process, which is the simplest way to generate nebulized gas from analytes dissolved in a liquid solution. Because the electric field applied in DMA is well defined, the collision cross sections are predictable and accurately defined, which makes DMA powerful for characterization and sizing distribution in a few minutes of bionanoparticles from 3 nm to several hundred nanometers.⁸⁸⁻⁹⁰ DMA has been successfully applied to characterize polymers, proteins, viruses, bacteriophages and nanoparticle-biomolecule conjugates.⁹⁰⁻⁹⁵

In this part, we are exclusively interested in the asymmetric field ion mobility spectrometry, DMS and FAIMS. To date, two devices are capable of performing asymmetric field or differential ion mobility spectrometry: the DMS and the FAIMS (which stands for field-asymmetric ion mobility spectrometry), commercialized by AB Sciex (SelexION technology) and Thermo Fisher Scientific, respectively. Although the geometry of the two devices differs (planar electrodes for DMS, cylindrical electrodes for FAIMS), the mechanism of separation remains the same.

4.2.4.2. Basics of asymmetric field / differential ion mobility spectrometry: the electric field dependence of the ion mobility and generation of the waveform

a) Definition of the differential ion mobility

In opposition to DT-IMS which operates in condition of low DC electric field as described previously, DMS and FAIMS use transverse high electric fields (> 10 kV/cm) and is referred as a non-linear ion transport.⁹⁶ While in conventional DT-IMS ions are distinguished and identified due to their low field mobility, DMS separates ions on the basis of their difference in their migration rates under high versus low field.⁹⁷⁻⁹⁸ This field dependence is

characterized by the so-called alpha function,⁹⁹ which is a characteristic ion property, similar to the ion mobility K in IMS or the m/z in mass spectrometry.⁵¹ At high electric fields, the mobility coefficient changes and the normalized field mobility dependence is defined as the following alpha function:

$$\alpha(E) = \frac{K(E)}{K(0)} - 1 \quad \text{Equation 3}$$

with: $K(0)$: mobility coefficient under low electric field conditions; E : electric field

The alpha function is stable and device independent, and changes with the variations of drift gas pressure,¹⁰⁰ temperature,⁵² humidity, and the nature and concentration of organic modifier added in the drift gas also plays a major role in the DMS separation of ion species, as discussed in § 4.2.4.3. *Chemical effects in the separation process of differential ion mobility spectrometry*.¹⁰¹⁻¹⁰²

b) DMS and FAIMS geometries

The differential ion mobility interface consists of two electrodes, usually spaced some millimeters from each other, by which a gas stream containing the ions passes. FAIMS uses cylindrical electrodes¹⁰³⁻¹⁰⁴ while in the DMS configuration, these electrodes are planar (i.e. 30 mm long x 10 mm wide with a 1 mm gap in-between; dimensions chosen to provide high transmission efficiency with the gas flow provided by the mass spectrometer). The two devices are similar in many aspects, including the principle mode of operation (see next section), but have distinct characteristics. The cylindrical geometry provides a higher resolution because the cylindrical fields better focus the ions at atmospheric pressure.¹⁰⁵ On the other hand, DMS has the advantage of faster scanning speeds and the ability to simultaneously transmit ions of both polarities and subject each to separation based on their differential mobility constants. In addition, DMS can be operated in transparent mode of operation, which allows all ions to be transmitted without discrimination when the separation voltage and compensation voltage are turned off.^{48, 106}

c) Generation of the RF field in DMS

In DMS, the ions are subjected to alternating high and low electric fields at high frequency. The asymmetric electric waveform, known as RF field or separation voltage (SV), is applied perpendicularly to the gas flow in-between the two parallel electrodes (Figure IV- 11.a). This SV consists of a short period of high field strength application of one polarity (t_{high} on Figure IV- 11.b) and then a longer period of low field of opposite polarity (t_{low} on Figure IV- 11.b). This results in the ions undergoing fast oscillations in response to this separation field. The ion's drift velocity now depends on the SV amplitude, the waveform and its intrinsic alpha function, which differs at high and low field. To avoid the neutralization of the ions toward one of the electrodes and correct the deviation of the drift trajectory, a low constant electric field, called compensation voltage (CoV, Figure IV- 11.a and c), is applied. The CoV is dependent on the separation field and the ion's alpha function, and is therefore specific to a

given ion.⁵² Thus, unlike in IMS where ions are separated in time, and instead of recording the flight time of an ion through the chamber, the voltage required to correct the trajectory of a particular ion is recorded for a range of SV amplitudes. The ion mobility coefficient is encoded in the compensation voltage used to correct the tilt in ion trajectory for each SV amplitude. As a result, it is necessary to scan the CoV at a constant SV value to allow all the ion species to reach the detector. This way, a full spectrum can be obtained.

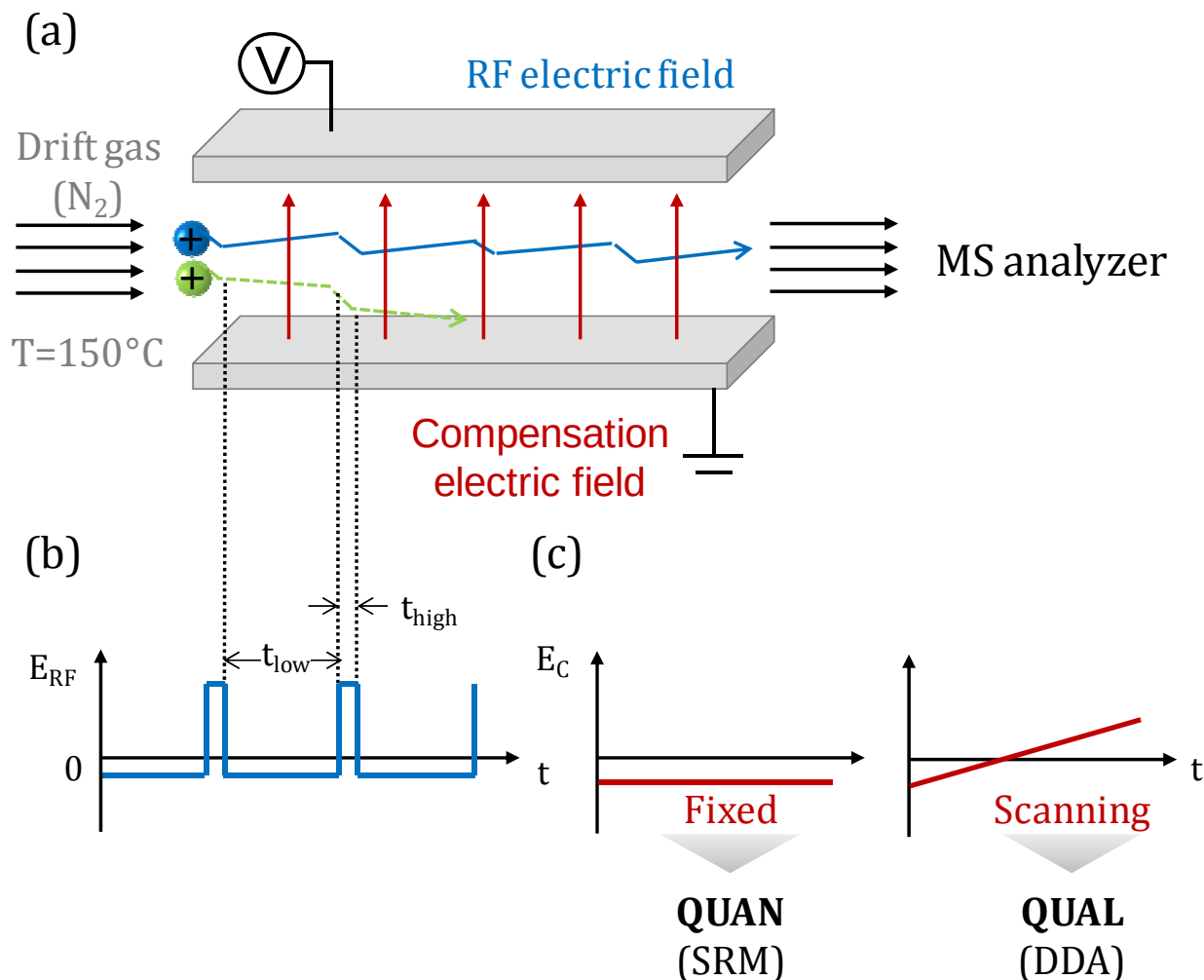


Figure IV- 11. Principle of differential mobility ion spectrometry. (a) General scheme showing the different electric fields applied in-between two electrodes; (b) asymmetric RF electric field = separation voltage; (c) uniform compensation voltage, operated either at fixed value or in scanning mode. The blue ion is selectively transmitted to the detector while the green ion is neutralized onto the electrode. Figure reproduced from Lambertus *et al.*¹⁰⁷

4.2.4.3. Chemical effects in the separation process of differential ion mobility spectrometry based on the dynamic cluster-decluster model

If the ion mobility K is relatively easy to calculate in IMS, the reason why an ion has different mobility characteristics under high field conditions versus low field condition is not well understood. To explain the physical nature of this field mobility dependence, Krylov and co-workers have proposed the theory of the clusterization model, also referred to as the

dynamic cluster-decluster model, as the most dominant effect affecting the DMS separation.⁵¹

A cluster is a bound state of an ion with neutral molecules, which can come from either drift gas molecules or organic modifier molecules (i.e. bound by non-covalent forces).⁵² Because these bounds are low energy bounds, the degree of clustering is strongly dependent on the electric field strength: i) at high electric fields, these bounds are broken and a declustering phenomenon is observed; ii) on the other hand, at low electric field, the clustering is predominant. This means that the difference in cluster mobility at low and high electric field strength is increased, which has the consequence of increasing the separation power of DMS. The energy involved in the formation and dissociation of the clusters is highly specific to the chemical interactions between ion and neutral gas-phase molecules. These interactions, which determine the mobility of the ion, are determined by collision dynamic parameters such as the size and shape of the collision partners and any electrostatic interactions that occur between them.¹⁰¹

The introduction of chemical modifiers (i.e. polar neutral molecules) in the DMS cell has been demonstrated to increase the separation power of DMS.^{49, 108-110} Therefore, Schneider *et al.* have reported the effect of several parameters having chemical effects on the DMS separation of ions, namely the effective temperature, the nature of the modifier and its concentration, and the nature of the gas transporting the ions.¹⁰¹⁻¹⁰² These parameters affect the dynamic equilibrium of the clustering process as follows:¹⁰²

- **Effect of the effective temperature**

Because the SV used in DMS varies between high field and low field regimes at a rate in the MHz range, this variation can be modeled as a field-dependent effective temperature synchronous with the SV field due to high collision frequency at atmospheric pressure. This will have an effect on the ion size, and thus change its ion-mobility cross-section, enhancing the differential mobility effect in DMS.⁵²

- **Influence of the concentration of the organic modifier**

It has been demonstrated that the molecular size of the cluster increases when the concentration of the organic modifier is increased, and experiments show that different modifiers produce different CoV values that cannot be predicted but that are reproducible (see ref.¹⁰² and Chapter 4, Part 3 of this thesis manuscript). However, while the peak capacity is improved by increasing the concentration of clustering modifier, ion losses may also increase as a function of the ratio of proton affinities for the modifier and the analyte of interest, hampering the signal for low proton affinity analytes. This can be advantageous to remove isobaric interferences by choosing a modifier of which proton affinity is lower than the analyte of interest but higher than the interfering species, but this requires prior knowledge of the sample composition beforehand.

- **Effect of the transport gas composition on the DMS separation and on the effective gap**

Because the separation mechanism in DMS is driven by the interactions of an ion and its clusters with the transport gas, the nature of this gas will affect the mobility, and therefore the peak capacity. The magnitude of the mobility of a particular ion in a particular transport gas will reduce the effective gap proportionally. The effective gap is defined by the degree to which there is a physical gap (i.e. physical distance between the two DMS electrodes). The oscillation amplitude creates a reduction of the effective gap. By definition, the effective gap is smaller than the physical gap because the ions need enough space to oscillate with the SV without being neutralized on the electrodes. The distance that an ion travels during each period of the waveform is affected by the masses (Equation 4) and cross-sections of the ion and transport gas as well as the interactions between (Equation 2).

$$K \propto \sqrt{\frac{m_1 m_2}{m_1 + m_2}} \quad \text{Equation 4}$$

with: K: ion mobility; m_1 : mass of the ion; m_2 : mass of the transport gas.

Ions travel further through low-mass nonpolar transport gases (e.g. helium) during a single waveform period because the mobility for a particular ion increases in light gases. As a result, low-mass ions, which have inherently high mobilities, will travel further through these gases and will strike the electrodes under electric field conditions that provide optimal separations for higher mass ions. Under extreme conditions, the effective gap can be zero.

It has been demonstrated that the peak resolution or resolving power (defined as $R_s = \text{CoV}_{\text{range}} / \text{FWHM}_{\text{average}}$; with FWHM: full-width at half maximum of the peak) improves as the fraction of He in the He/N₂ mixture increases to 50% (v/v). The gain generally comes from both broader separation space (greater distance between the peaks) and also because the ions are more mobile in lighter gases, which narrows the peaks.¹¹¹

With helium, low-mass ions can be lost to the DMS electrodes, and particularly when high separation voltages are applied. The net effect is similar to reducing the gap dimensions.

If the first FAIMS/DMS studies have reported resolving power no better than 20, stronger electric fields and optimized gas mixtures (i.e. up to 75-85% of He in the He/N₂ mixture) have recently enabled to achieve resolving power up to 200.¹¹²⁻¹¹³ Together with improvements in instrumentation where fluctuations of the voltages and gas pressure have become critical, and also by using H₂/N₂ gas mixtures, high-definition DMS can be achieved with resolving power up to 500.¹¹⁴

- **Formation of heterogeneous clusters from electrospray ionization**

The incomplete electrospray desolvation may lead to the formation of heterogeneous clusters of different sizes and compositions in the residual solvent droplets. This will lead to a consequent broadening of the peak widths in the DMS spectrum. However, the accurate characterization of the heterogeneous population is difficult for several reasons: they may

not survive the transport process into the MS analyzer; the bond energies of typical clusters with non-polar molecules are so low that they do not survive intact during the transport from atmospheric conditions to the vacuum system; finally, collisions in the free jet expansion are energetic enough to disrupt these clusters to the bare molecular ion. This is emphasized when the device is hyphenated in between a liquid chromatographic system and an ESI-mass analyzer, and especially at higher flow rate than 100 $\mu\text{m}/\text{min}$, where the heterogeneous cluster ion population is related to the mobile phase introduction flow rate. This can be controlled by increasing the temperature of the nebulized electrospray, to improve the desolvation efficiency.¹⁰² As a consequence, ESI sources operating at liquid flow rates within the nL/min to low $\mu\text{m}/\text{min}$ produce fewer clusters.

- **Control of the temperature and pressure affecting the collision parameters**

Because the gas density is dependent on temperature and pressure creating the DMS cell environment, both play a role in the number of collisions of ions with gas molecules.^{52, 100, 115} The temperature of the transport gas is inversely proportional to the gas density and this influences the chemical interactions occurring, which shift the equilibrium in one direction or the other. In addition, as the ambient conditions cannot be controlled for obvious reasons, variations in weather conditions may be sufficient to affect the DMS separation.

- **Consequences on the DMS spectrum**

There are two effects of dynamic ion-neutral cluster formation change on the DMS spectrum: the variation of peak position in the CoV space, and the modification of the peak width. The latter is explained as follows: when cluster formation is important, the low field portion will have a higher average cluster number and a wider cluster distribution than the high field portion; this results in dispersion in the differential mobility and thus an increase in peak width.¹⁰² This is illustrated and discussed in the 3rd part of this chapter.

4.2.4.4. Applications

Ion mobility spectrometry (IMS) has become an important method for the detection of many compounds because of its high sensitivity and amenability to miniaturization for field-portable monitoring; applications include detection of narcotics, explosives, and chemical and biological warfare agents.¹¹⁶ FAIMS/DMS have found applications for various compounds from small molecules (e.g. pharmaceuticals,^{48, 117} drugs of abuse,¹¹⁸ lipids^{47, 119}) to peptides (e.g. analysis of tryptic digests¹¹²) or proteins (e.g. to investigate oligomer assembly and protein conformation changes¹²⁰). FAIMS/DMS have disabled the separation of isomers such as small amino acids leucine and isoleucine (i.e. by electrospray-FAIMS-MS⁶⁴) and also the removal of isobaric interferences.¹²¹ DMS has also proven its suitability after being used in combination with a direct surface analysis technique such as DESI, for the analysis of diverse samples including counterfeit pharmaceuticals and binary mixtures

of isobaric chemicals of importance in the pharmaceutical and food industries. The combination between DESI and DMS has led to a considerable increase in selectivity and sensitivity (i.e. S/N improved up to 70-90%).¹²² It has also been used in combination gas chromatography-MS for the rapid determination of alcohols in human saliva following selective membrane extraction¹²³ or for the detection and quantification of natural contaminants of wine.¹²⁴

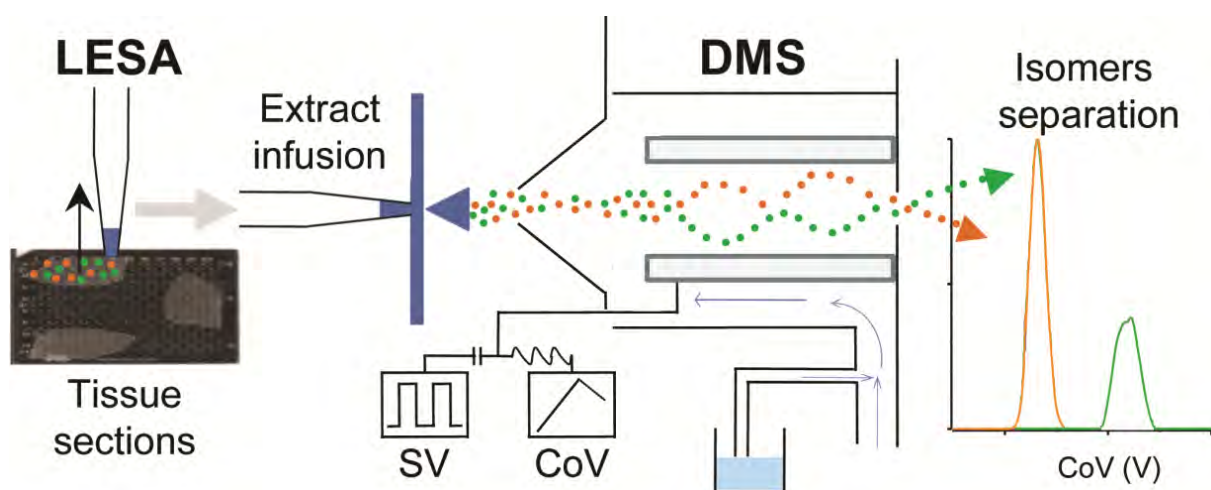
4.2.4.5. Perspectives for DMS

Because DMS operates at ambient temperature, and can be easily used in combination with ESI source or even nES source, DMS has been selected as a potential tool to be used together directly after a surface sampling technique such as those employing liquid microjunction.¹²⁵ The application of this new analytical platform combining liquid extraction surface analysis (LESA) and differential ion mobility spectrometry (DMS) on a triple quadrupole linear ion trap QTRAP 5500 platform is presented within the third part of the Chapter 4 of this manuscript.

CHAPTER 4.

Development and application of LESA-DMS-MS/MS

Part 3 – Gas-phase separation of Drugs and Metabolites using Modifier-Assisted Differential Ion Mobility Spectrometry Hyphenated to Liquid Extraction Surface Analysis and Mass Spectrometry



Adapted from:

T. Porta, E. Varesio and G. Hopfgartner, *Anal. Chem.* **2013**, 85 (24), 11771–117793

Abstract

The present work describes an alternative generic approach to LC-MS for the analysis of drugs of abuse as well as their metabolites in postmortem tissue samples. The platform integrates liquid extraction surface analysis (LESA) for analytes tissue extraction followed by differential ion mobility spectrometry (DMS) mass spectrometry for analytes gas phase separation. Detection is performed on a triple quadrupole linear ion trap using the selected reaction monitoring mode for quantification as well as product ion scan mode for structural confirmatory analyses. The major advantages of the platform are that neither chromatographic separation nor extensive sample preparation are required. In DMS the combination of a high separation voltage (i.e. up to 4 kV) together with organic modifiers (e.g. alcohols, acetonitrile, acetone) added in the drift gas is required to achieve the separation of isomeric metabolites, such as the ones of cocaine and tramadol. DMS also separates morphine from its glucuronide metabolites, which allows for preventing the overestimation of morphine in case of fragmentation of the glucuronides in the atmospheric-to-vacuum interface of the mass spectrometer. Cocaine, opiates, opioids, amphetamines, benzodiazepines and several of their metabolites could be identified in postmortem human kidney and muscle tissue based on simultaneous screening and confirmatory analysis in data-dependent acquisition mode using an analyte-dependent compensation voltage to selectively transmit ions through the DMS cell to the mass analyzer. Quantitative performance of the LESA-DMS-MS platform was evaluated for cocaine and two of its metabolites spotted onto tissue sections using deuterated internal standard. Analyte responses were linear from 2 to 1'000 pg on tissue corresponding to a limit of detection on the order of 1 ng of analyte per gram of tissue. Accuracy and precision based on QC sample was found to be less than 10%. Replicate analyses of cocaine and its metabolites in forensic samples showed an intra- and inter-section variability of less than 25%.

Key words

Differential ion mobility, liquid extraction surface analysis, mass spectrometry, forensic toxicology, metabolites.

4.3.1. Introduction

In drug metabolism or forensic toxicology, liquid chromatography with mass spectrometric detection (LC-MS) has become the technique of choice for performing sensitive and selective analysis of drugs and their metabolites in complex biological samples.¹²⁶ While toxicological screening is mainly performed in urine or blood, the analysis of alternative specimens such as tissues (e.g. kidney, liver or muscle) is often required to confirm or exclude the ingestion of drugs.¹²⁷ However, LC-MS analysis of drugs in tissue remains challenging,¹²⁸ because the development of appropriate sample preparation requires multiple-steps such as mechanical homogenization, extraction or digestion. The sample preparation may last up to several hours, increasing dramatically the overall analysis throughput. Variable extraction recovery of the analytes from the tissue homogenates is also a limitation for quantitative analysis.¹²⁹

In the past few years, direct surface analysis (or direct tissue profiling), including matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI) and secondary ion mass spectrometry (SIMS), was found particularly attractive in detecting drugs and metabolites in tissues because it maintains sample spatial integrity. The more representative examples are drug tablets and powders,¹³⁰ explosives,¹³¹ inks,¹³² urine,¹³³ fingerprints,¹³⁴⁻¹³⁵ tissue sections¹³⁶⁻¹³⁷ and single hair samples.¹³⁸⁻¹³⁹

Compared to tissue homogenization procedures, direct tissue profiling is limited to a single sample preparation step of tissue sectioning, and for MALDI-based analyses an additional deposition of the matrix. Moreover, only a small part of the tissue section is sampled and additional analyses are possible at any time on the rest of the tissue section, which is of utmost importance in forensics when additional investigations are requested. Recently, liquid extraction surface analysis (i.e. LESA), also referred to as liquid micro-junction surface sampling probe²⁴ has been introduced to map the distribution and to study the metabolism of low molecular weight drugs in order to complement whole body autoradiography (WBA).²⁵ As in most direct surface analysis techniques, LESA directly coupled to the mass spectrometer cannot separate in single MS mode analytes with identical elemental composition or even by tandem MS in the case of certain metabolites. To circumvent this limitation, the coupling of a liquid chromatographic system subsequent to the liquid extraction surface sampling^{42, 44} to separate those compounds has been described. An alternative to chromatography is to use ion mobility separation (IMS) that offers an orthogonal mechanism of resolving isomeric analytes. Indeed, IMS separates ions in the gas-phase on the basis of their collisional cross-section prior to their separation according to their mass-to-charge ratio by the mass analyzer. Among the existing ion mobility techniques, differential ion mobility spectrometry (DMS),⁹⁹ also known as field asymmetric waveform ion mobility spectrometry (FAIMS), has shown its potential to reduce chemical noise and removal of interferences in atmospheric pressure ionization mass spectrometry,^{106, 140-141} which is particularly attractive for quantitative LC-MS. DMS has been used to resolve isomeric exogenous metabolites,¹⁴² as well as for forensic investigations.^{118, 123} Recently,

Krylov *et al*^{51, 101, 143} have shown that the addition of an organic modifier to the drift gas enhances the DMS separation power by increasing its peak capacity (i.e. modifier-assisted DMS). They report that clusters between the analyte ions and the neutral molecules of the modifier are preferentially formed in the low electric field portion, whereas a declustering phenomenon occurs in the high electric field portion, which considerably increases the difference between low-field and high-field analyte's ions mobilities.

The objective of the present work is to evaluate a new integrated platform based on the hyphenation of LESA and DMS with a triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer for the direct analysis of drugs of abuse and some of their metabolites in tissue sections. The first part of the work investigates the gas-phase separation performance of differential ion mobility enhanced by the use of organic modifiers for a set of 30 drugs of abuse including isomeric metabolites of cocaine, tramadol and morphine. The second part demonstrates the application of the LESA-DMS-MS platform in forensic investigations of postmortem human kidney and muscle tissue sections. Finally, the potential use of the LESA-DMS-SRM/MS for quantitative analysis, considering limits of detection and dynamic range, is evaluated for cocaine and its metabolites in tissues.

4.3.2. Experimental Section

4.3.2.1. Chemicals and reagents

Cocaine (COC), benzoylecgonine (BZE), ethylcocaine (ECOC), ecgoninemethylester (EME), morphine (MOR), morphine-3-beta-glucuronide (M3G), morphine-6-beta-glucuronide (M6G), codeine, amphetamine (A), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), d,l-methadone, olanzapine (OLZ), fentanyl citrate, lorazepam (LZ), midazolam (MDZ) and diazepam (DZ) were provided by Lipomed (Arlesheim, Switzerland). Norcocaine (NCOC), *cis*-tramadol (TRMD), O-desmethyl-*cis*-tramadol (ODT), N-desmethyl-*cis*-tramadol (NDT), trimipramine, N-desmethyl-trimipramine, lorazepam glucuronide (LZ-glu), were provided by Cerilliant (Molsheim, France). Promazine and chlorprothixene were supplied by LGC Standards (Teddington, Middlesex, UK). N-desmethyloanzapine (N-desmethyl-OLZ) was provided by BDG Synthesis (Wellington, New Zealand). Nordiazepam (NorDZ) was a gift from the Geneva University Hospital (Dr. Fathi, Geneva, Switzerland). Methanol (MeOH, HPLC grade), acetonitrile (ACN, HPLC grade), isopropanol (IPA) and ethanol (EtOH) were provided by VWR International (Nyon, Switzerland). Acetone (HPLC grade) was purchased from Sigma-Aldrich (Buchs, Switzerland). Formic acid (HCOOH) was provided by Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

A stock solution containing the set of 30 drugs and metabolites was prepared at a concentration of 10 µg/mL in MeOH and stored at 4°C prior the analysis. A mixture was prepared in MeOH:H₂O:HCOOH (60/40/0.1, v/v/v) at a final concentration of 250 ng/mL

and was used for infusion. Standard solutions were then prepared at concentrations ranging from 2 to 1'000 ng/mL and were spotted (1 μ L) either onto a stainless steel plate or onto tissue sections when specified for quantitative purposes.

4.3.2.2. Postmortem tissue samples

Postmortem kidney and muscle human samples were provided by the Department of Forensic Pharmacology and Toxicology (Institute of Legal Medicine) of the University of Zurich. As handling human tissue material is of potential risk, precautions taken during manipulations and proper recycling of these samples require appropriate personnel training and safety equipment. **Preparation of tissue sections.** Muscle and kidneys were ablated postmortem and then directly ice-frozen and stored at -80°C prior the analysis to prevent postmortem degradation of drugs of abuse.¹²⁷ Frozen kidney was then heated to -20°C, cut at 12 μ m thickness using a cryostat (Leica CM3050 S, Leica Microsystems, Wetzlar, Germany) and thaw-mounted onto a stainless steel plate. **Direct quantification of tissues.** The solution containing the set of analytes was spotted at different locations onto blank tissue sections with increasing concentration from 2 to 1'000 ng/mL. Deuterated analogues (i.e. d3-standards) were used as internal standards and spotted (1 μ L) at a concentration of 250 ng/mL on the top of the different spots previously deposited.

4.3.2.3. Sample preparation for the quantification of drugs of abuse from tissue homogenates by LC-MS. Tissue samples

100 $\pm$ 5 mg of each tissue sample were weighed using a Mettler AE420 balance (Mettler Toledo, Greifensee, Switzerland). To limit sample handling, samples were not dried prior to weighting. **Drug solutions for calibration.** Standard solutions containing a cocktail of 16 drugs (COC, BZE, NCOC, ECOC, MOR, M3G, M6G, DZ, NorDZ, TRMD, ODT, NDT, MTD, OLZ, OLZ-metabolite, PRMZ) were prepared in MeOH/H₂O (20/80, v/v) at concentrations ranging from 10 to 20'000 ng/mL. A mixture of 5 deuterated standards (COC-d3, MOR-d3, DZ-d5, TRMD-d3, MTD-d10) was prepared at a concentration of 5 μ g/mL also in MeOH/H₂O (20/80, v/v). Calibrations curves were built using 10 standards of calibration (CAL). The concentrations of the spiking solutions were of: 10, 25, 50, 100, 250, 500, 1'000, 5'000, 10'000 and 20'000 ng/mL. **Preparation of the calibration curves and quality control samples from bovine tissue (kidney) homogenates.** Calibration and quality control (QC) samples were prepared using bovine kidney as follow: to 100 mg of tissue weighted in a 1.5 mL Eppendorf tube, 5 μ L of spiking solution + 5 μ L of ISTD (5 μ g/mL) were added. The resulting calibration curves were then built at an analyte's concentration per gram of tissue ratio ranging from 0.5 to 1'000 ng_{analyte}/g_{tissue}. The Eppendorf tubes were then closed and frozen in LN₂ for 1-2 minutes. A 5 mm-diameter stainless steel bead was then added in each tube. Samples were homogenized using a Mixer Mill MM400 apparatus (Retsch GmbH, Haan, Germany) operated at 30 Hz for 4 minutes. 200 μ L of ACN/EtOH (1/1, v/v) were then added

and samples were mixed again at 30 Hz for 5 minutes with the MM400. Samples were finally centrifuged at 12'000 rpm for 10 minutes and the supernatants were taken off before being evaporated to dryness. Samples were then reconstituted in 50 μ L of ACN/H₂O/HCOOH (20/80/0.1, v/v/v) and then diluted 10 times in the same solvent composition for further LC-MS analysis for quantification. Precision and accuracy were calculated based on 10 QC samples covering whole calibration range. ***Preparation of forensic samples for quantification of drugs of abuse.*** 100 mg of postmortem human kidneys were treated as previously described, except that no spiking solution was added. Each forensic sample was extracted 5 times from 5 independent weightings.

4.3.2.4. Liquid chromatography mass spectrometry

The LC-MS separation of the set of drugs of abuse and some of their metabolites (250 ng/mL each) was performed on a LCMS 8040 MS instrument hyphenated with a low pressure gradient pump (Nexera, Shimadzu, Kyoto, Japan). A reversed-phased column (Kinetex 1.7 μ m XB-C18, 100 A, 2.1 x 100 mm, Phenomenex, Torrance, CA, USA) was used for the separation. The mobile phases were: A: H₂O+0.1%HCOOH and B: MeOH+0.1%HCOOH (flow rate of 300 μ L/min). The analysis was achieved in 10 minutes in gradient mode as follow: 0%B for 0.5 min; from 0%B to 75%B (linear) over 5 min, followed by a 1 min column washing (95%B) and 3.5 min re-equilibration (0%B). Acquisitions were performed in full scan MS mode, and isomers were identified by injecting each compound individually. Operating conditions were: mass range: m/z 50-700 (scan speed of 7500 u/s); spray voltage: 4.5 kV; heat block temperature: 400°C; nebulizing gas flow: 3 L/min; drying gas: 15 L/min.

4.3.2.5. LESA-DMS-MS platform

The LESA-DMS-MS platform is shown in Figure IV-12 and described hereafter. ***Liquid extraction surface analysis (LESA).*** LESA was performed directly on tissue sections using a chip-based nanoelectrospray (nES) system (TriVersa NanoMate, Advion BioSciences, Harlow, UK), used as the ionization source. MeOH:H₂O:HCOOH (75:25:0.1, v/v/v) was used as solvent for liquid extraction. Operating conditions were: aspirated solvent: 2 μ L, dispensed solvent: 1 μ L for 10 seconds, post-aspirated solvent: 1.5 μ L. The nES was generated by applying a 0.4 psi gas pressure (nitrogen) and a 1.55 kV voltage. The method also includes an additional time in case the infusion fails and the robot has to automatically change the position of the pipette tip to the nozzle on the nanoES chip. ***Differential ion mobility and mass spectrometry (DMS-MS).*** Experiments were performed in positive ionization mode on a QTRAP 5500 mass spectrometer equipped with a prototype DMS cell placed in front of the orifice plate (AB SCIEX, Concord, ON). Organic modifiers were introduced in the cell at a concentration of 1.5% in the curtain gas (N₂, 3 l/min, 10 psi) using a continuous pumping system (Agilent 1100 series quaternary pump, Agilent Technologies,

Waldbronn, Germany) at the following flow rates: 109.5 $\mu\text{L}/\text{min}$ (ACN), 84.5 $\mu\text{L}/\text{min}$ (MeOH), 121.8 $\mu\text{L}/\text{min}$ (EtOH), 152.9 $\mu\text{L}/\text{min}$ (acetone) and 159.5 $\mu\text{L}/\text{min}$ (IPA). DMS operating conditions were: cell temperature = 150 $^{\circ}\text{C}$; resolution = standard; offset = -6 V; separation voltage (SV) = 4'000 V. Experiments were performed either at a fixed compensation voltage (CoV) for quantitative analyses in the selected reaction monitoring (SRM) mode (details in Tables IV-1 and IV-2) or at a varying CoV value (from -60 V to 10 V, steps of 0.2 V) for the optimization of the separation and the structural identification in the data-dependent acquisition (DDA) mode. For DDA experiments, the survey scan consisted of ramping the CoV value while monitoring the SRM transitions. Triggered MS/MS dependent experiments were acquired in enhanced product ion (EPI) mode with the following conditions: m/z mass range of 50 – 500 (scan speed of 1'000 u/s), trap fill time set at 250 ms and a collision energy of 35 eV with a spread of ± 15 eV (collision gas = nitrogen). The Q_1 quadrupole was operated at unit resolution for the precursor ions selection.

4.3.2.6. Software for data acquisition and processing

ChipSoft Manager software (v.8.3.2.1209, Advion BioSciences) was used to control automatic liquid extraction from surface sample with the TriVersa NanoMate. Analyst 1.5 software (AB SCIEX) was used for mass spectrometer control and data collection. A dedicated driver provided by Advion BioSciences was used to create and launch the batches. PeakView software (v. 1.0, AB SCIEX) was used for data processing. MultiQuant software (v. 2.0, AB SCIEX) was used for processing of quantitative data by LC-SRM/MS. CoV values were imported into R software (v. 3.0.1)¹⁴⁴ to generate scatterplots using the ggplot2 package.¹⁴⁵

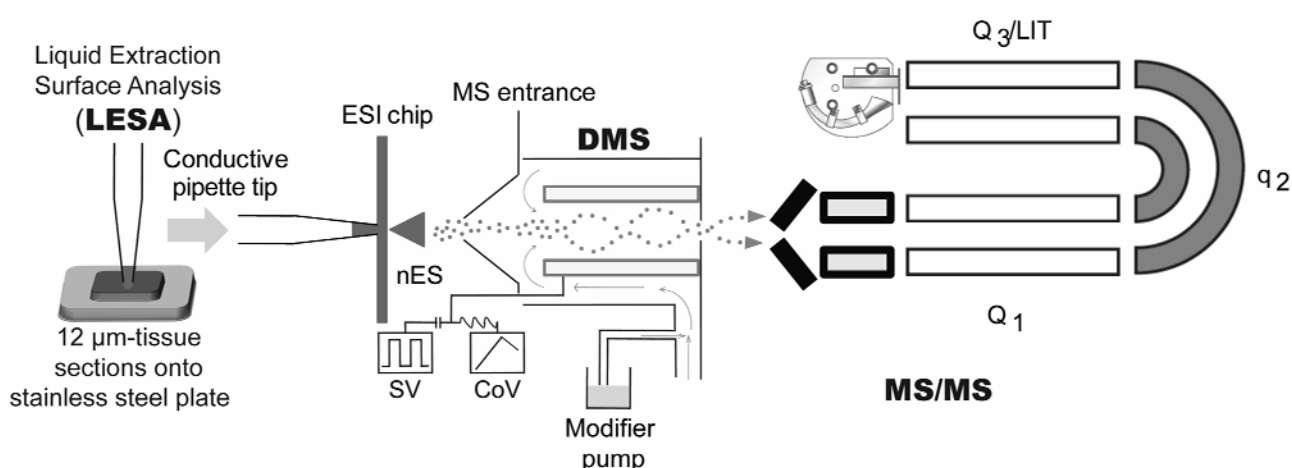


Figure IV- 12. Analytical platform for LESA-DMS-MS/MS analyses. nES = nanoelectrospray. SV = separation voltage. CoV = compensation voltage. Scheme not to scale. Q1, q2, Q3 and D refer to the first quadrupole, the CID collision cell, the third quadrupole and the detector of the mass analyzer, respectively.

Table IV- 1. SRM transitions monitored. The dwell time was set to 10 ms for each SRM transition

<i>Analytes</i>	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	CE (eV)	DP (V)
DZ	285.0	193.0	45	120
NorDZ	271.1	140.0	41	120
LZ	321.0	275.0	32	100
LZ-glucuronide	497.0	321.0	21	60
MDZ	326.0	291.1	40	110
OLZ	313.1	256.1	31	100
4-desmethyl-OLZ	299.0	256.1	36	100
MOR	286.1	201.1	36	100
M3G/M6G	462.1	286.1	45	100
Codeine	300.1	165.1	66	100
TRMD	264.2	57.8	55	55
ODT	250.1	57.8	52	60
NDT	250.1	44.0	40	60
COC	304.1	182.2	28	65
BZE/NCOC¹	290.1	168.1	28	70
NCOC²	290.1	136.1	35	70
ECOC	318.1	196.1	30	70
EME	200.1	182.2	26	70
A	136.1	91.1	30	35
MA	150.1	91.0	32	45
MDMA	194.1	163.1	18	55
MDEA	208.1	163.1	20	55
MDA	180.1	163.1	15	45
Promazine	285.1	86.0	25	60
Methadone	310.1	265.1	23	60
Trimipramine	295.1	100.0	23	70
N-desmethyl-trimipramine	281.1	86.0	23	70
Chlorprothixene	316.0	271.0	30	90
Fentanyl	337.2	188.2	32	80
<i>Internal standards</i>				
COC-d3	307.1	185.2	28	70
A-d5	141.1	92.9	30	35
TRMD- ¹³ C-d3	268.2	58.0	57	50
MOR-d3	289.1	201.1	35	100
Diazepam-d5	290.1	198.1	48	100
Trimipramine-d3	298.2	102.2	35	70

¹: most intense transition common to BZE and NCOC. ²: second most intense SRM transition specific to NCOC.

Table IV- 2. Compensation voltage values obtained without or with the different modifiers at 1.5% in the drift gas. Data for ACN are given with relative standard deviation (RSD) obtained from 4 infusions performed over a period of several months.

Compounds	No modifier	IPA	Acetone	EtOH	MeOH	ACN		
	r	SV=4.0 kV				SV=4.0 kV		SV=3.5 kV
		CoV (V)				CoV (V)	RSD (%)	CoV (V)
DZ	5.8	-19.7	-14.0	-17.8	-13.5	-12.1	4.6%	-9.8
NorDZ	4.7	-37.0	-27.6	-27.1	-19.7	-23.9	0.8%	-18.0
LZ	5.9	-35.9	-19.4	-29.1	-18.7	-21.7	1.7%	-14.3
LZ-glucuronide	6.0	-29.8	-18.8	-17.8	-8.8	-15.7	1.6%	-11.2
MDZ	6.6	-13.0	-8.5	-14.3	-11.3	-7.1	4.6%	-6.2
OLZ	6.9	-26.4	-22.6	-7.1	-0.3	-19.6	0.8%	-13.6
4-desmethyl-OLZ	6.6	-28.6	-25.1	-7.6	-0.7	-21.6	1.1%	-15.2
MOR	4.5	-30.0	-29.4	-21.4	-16.9	-21.4	3.1%	-16.5
M3G/M6G	5.8	-17.6	-15.5	-10.8	-7.4	-10.0	3.2%	-7.8
M3G/M6G*	n.s.	n.s.	n.s.	-7.6	-3.9	n.s.	n.s.	n.s.
Codeine	4.9	-22.9	-20.4	-18.6	-14.8	-16.9	2.3%	-13.3
TRMD	7.8	-19.4	-32.8	-1.5	2.6	-30.8	0.6%	-22.9
ODT	6.8	-32.1	-46.6	-7.8	-1.0	-36.2	0.7%	-26.6
NDT	5.0	-27.5	-24.9	-22.3	-14.5	-20.8	1.0%	-16.2
COC	7.3	-13.3	-29.4	0.1	3.2	-24.4	0.7%	-17.7
BZE	6.0	-21.8	-27.5	-19.5	-12.9	-27.0	1.3%	-18.6
NCOC	6.5	-21.5	-18.1	-16.8	-13.2	-14.4	4.3%	-11.9
ECOC	7.9	-10.6	-26.9	-1.4	4.2	-22.0	0.8%	-16.0
EME	5.8	-32.4	-58.3	-7.9	-1.7	-47.8	1.0%	-35.5
A	-1.7	-51.0	-26.3	-55.1	-41.8	-30.3	1.5%	-21.0
MA	2.2	-54.5	-27.5	-51.0	-36.4	-30.0	3.0%	-19.6
MDMA	2.3	-39.8	-16.3	-37.7	-27.3	-18.7	1.9%	-11.9
MDEA	4.5	-39.8	-22.2	-36.1	-25.0	-22.6	1.2%	-15.7
MDA	-0.1	-39.8	-17.6	-41.4	-32.0	-21.5	1.2%	-14.9
Promazine	7.5	-3.8	n.a.	-3.8	-7.7	-25.5	0.8%	-19.5
Methadone	8.1	0.1	-20.9	5.5	6.3	-15.5	3.9%	-12.7
Trimipramine	7.7	-6.8	-27.8	3.1	5.1	-22.3	0.9%	-16.6
N-desmethyl-trimipramine	6.5	-12.3	-14.4	-12.3	-8.4	-12.0	1.0%	-8.9
Chlorprothixene	7.1	-18.1	-19.4	-14.4	-6.6	-16.1	1.4%	-11.7
Fentanyl	9.4	-28.0	-32.1	-6.3	0.8	-25.1	1.8%	-19.4

* second peak for morphine glucuronide metabolites when separated. n.s.: not separated. n.a.: not available.

4.3.3. Results and Discussion

4.3.3.1. Parameters influencing the DMS gas-phase separation and the MS response

Although the asymmetric RF voltage (i.e. the separation voltage, SV) was applied between the two planar electrodes, the drift gas type and the cell temperature play major roles in the DMS separation, it has been recently demonstrated that the addition of an organic modifier in the drift gas also considerably increases the separation power of the DMS.^{143, 146} Therefore, with a DMS cell temperature of 150°C and nitrogen as drift gas, the effects of the SV amplitude and the nature of the modifier were evaluated for the separation of a set of 30 drugs of abuse and metabolites from different classes (e.g. amphetamines, cocaine, opiates, opioids, benzodiazepines).

a) Equations used to assess the separation performance

Calculation of peak capacity and resolution between the three pairs of isomeric metabolites was used to assess DMS separation performances. The peak capacity (PC) defines the maximum number of components that can be separated per unit of resolution within a given CoV range (i.e. [CoV_{max} – CoV_{min}]). It is calculated as follows:

$$PC = \frac{(CoV_{max} - CoV_{min})}{1.7 \times FWHM_{averages}} \quad \text{Equation 1}$$

where CoV is the compensation voltage and FWHM_{averaged} is the full width at half maximum averaged over the 30 analytes. The resolution (Rs) between two isomeric peaks was calculated according to Equation 2.

$$Rs = 1.18 \times \frac{|CoV_1 - CoV_2|}{FWHM_1 + FWHM_2} \quad \text{Equation 2}$$

The separation between two peaks is considered complete for a resolution greater than 1.5, where there is only less than 2% of overlap between two Gaussian peaks of equal area.

b) Effect of the SV amplitude and nature of the organic modifier on peak capacity

When raising the separation voltage from 1.5 to 4.0 kV, peak capacity will normally increase (a typical example is shown in Fig. S-1a for the separation of five amphetamines). This gain in peak capacity is however not always sufficient to separate isomeric compounds, such as benzoylecgonine (BZE) and norcocaine (NCOC) that are both cocaine metabolites and share the same intense SRM transition (Fig. S-1b). When adding an organic modifier to the drift gas, the peak capacity can be dramatically altered (Fig. S-1c). For example, when using ACN as a modifier, an SV value greater than 3.4 kV already gives a resolution between the two isomers higher than 1.5 with a maximum Rs value of 3.95 at 4.0 kV (Fig. S-1d). In order to maximize peak capacity, further experiments are performed at the maximum SV amplitude of 4.0 kV, which is the highest stable voltage before the onset of electrical arcing into the DMS cell under these experimental conditions (i.e. drift gas nature, cell temperature, etc.).

The increased peak capacity offered by modifier-assisted DMS was investigated for the set of 30 drugs of abuse and some of their isomeric metabolites by introducing different organic modifiers (i.e. MeOH, EtOH, IPA, acetone and ACN) into the drift gas (nitrogen). While different CoV values were obtained for each modifier, strong correlations were observed between some pairs of modifiers (e.g. MeOH vs. EtOH or ACN vs. acetone) or between a modifier and nitrogen alone. As a matter of fact, the use of alcohols as modifier (red box in Fig. S-2) results mostly in a translation of the analytes CoV values. The same behavior is observed when exchanging ACN for acetone (blue box in Fig. S-2), but when replacing ACN or acetone for an alcohol as modifier, the selectivity (i.e. the peaks order) is considerably altered and no correlation is observed. Figure 2 shows selected examples of the DMS separation of the DoA obtained without or with three different modifiers (i.e. ACN, acetone and MeOH) added at 1.5% in the drift gas (nitrogen).

With the addition of ACN (Fig. IV- 13.b), acetone (Fig. IV- 13.c), MeOH (Fig. IV- 13.d), EtOH or IPA, the peak spread in the CoV scale is considerably increased compared to nitrogen alone (Fig. 2a). Optimal CoV values are recorded at the apex and are listed in Table IV-2 for each analyte/modifier combination. Figure IV- 14 shows that peak capacity is increased by about a 3-fold factor (i.e. 2.7- to 3.4-fold) when adding IPA, ACN and acetone and by 3.8- to 4.4-fold when using MeOH or EtOH, respectively. The use of organic modifiers does not increase significantly the peak widths (i.e. FWHM is affected by 1.2- to 1.3-fold), except for IPA where an average increase of 1.8-fold is observed.

Although the addition of modifier to the drift gas alters selectivity and enhances peak capacity, a loss of absolute MS response is generally observed for all of the compounds compared to nitrogen alone (Fig. S-3). The use of IPA gives the maximum loss of MS signal (i.e. only 0-25% of the signal is recovered compared to nitrogen alone for most analytes), whereas ACN and MeOH are the modifiers leading to a minimum loss of sensitivity (i.e. most of the analytes give a signal within 25-75% of the signal measured without modifier and up to 75-125% of the signal is observed for the group of amphetamines). MeOH appears particularly well suited for the analysis of amphetamines (A, MA, MDA, MDMA and MDEA) because peak intensities measured for these analytes are within 80 to 120% of intensities measured for the same analytes without a modifier.

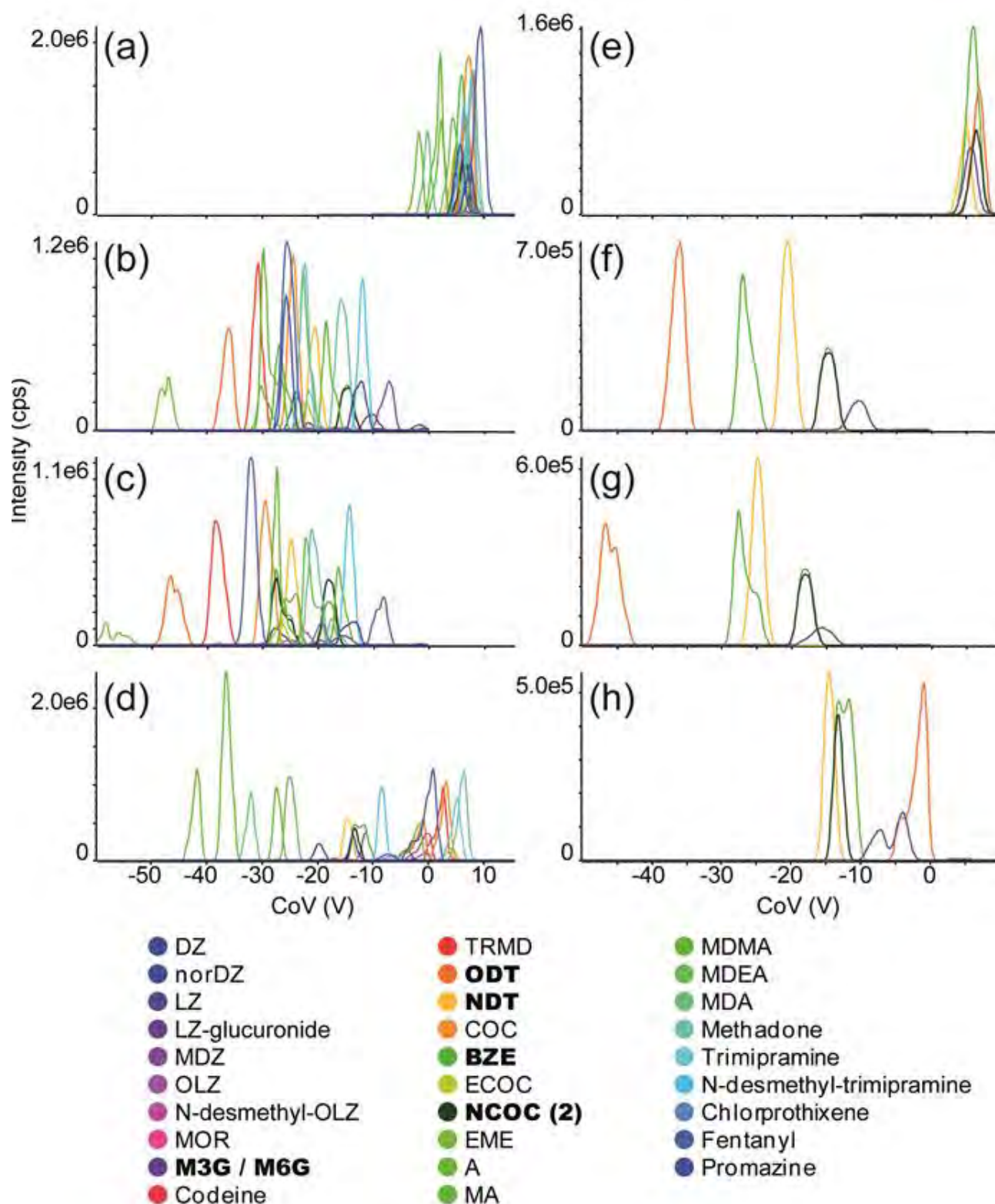


Figure IV- 13. Effect of three organic modifiers on the separation of a set of 30 drugs of abuse and some of their isomeric metabolites. (a-d) XIC of all SRM traces; (e-h) XIC of the three pairs of isomeric metabolites: M3G/M6G (dark blue), BZE (light green) / NCOC (dark green) and ODT (yellow) / NDT (orange). Conditions: SV = 4'000 V; (a,e) no modifier (nitrogen); 1.5% of (b,f) ACN; (c,g) acetone and (d,h) MeOH added to the drift gas.

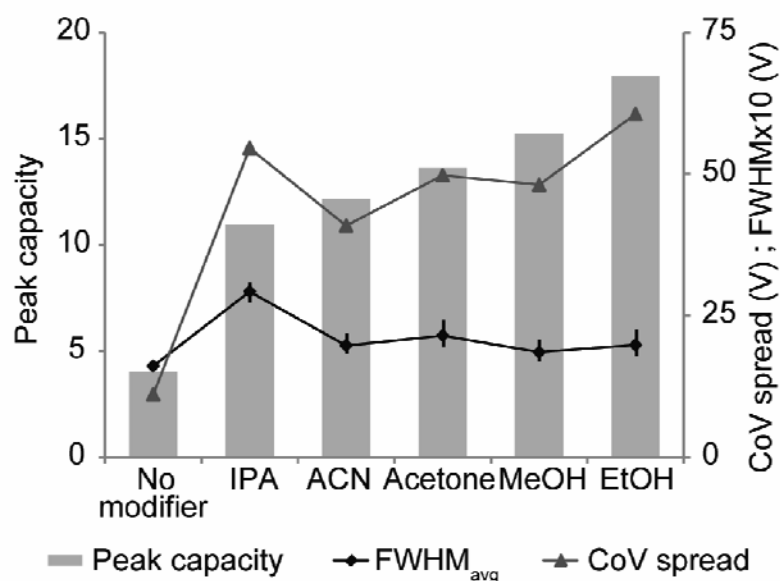


Figure IV- 14. Effect of the organic modifiers on peak capacity, CoV spread and average full width at half maximum (FWHM, confidence interval at 95%, n=30).

4.3.3.2. Separation of isomeric metabolites of cocaine, morphine and tramadol

In forensic toxicology, the determination of parent drugs and their metabolites is of utmost importance for distinguishing between illegal consumption of prohibited drugs and external contamination, which may lead to false positive results.

There are some cases where the differentiation between metabolites is mandatory. For instance, COC can be easily hydrolyzed in BZE or ecgonine methyl ester (EME), even from an external source of contamination. The detection of metabolite(s) formed by another pathway is required to conclude a drug intake. This is the case for instance for cocaethylene (ECOC), a toxic metabolite formed in the presence of ethanol (i.e. traducing the simultaneous consumption of alcohol) or NCOC, formed by N-demethylation of COC. BZE and NCOC are two isomeric metabolites with the same elemental composition. The MS/MS fragmentation of their precursor ion (m/z 290) results in the same most intense fragment at m/z 182. Consequently, by monitoring the SRM transition m/z 290 > m/z 168 and without chromatographic separation, one cannot differentiate the two metabolites and may under- or over-estimate their response. A second transition specific to NCOC can be monitored, but at the expense of the assay sensitivity since this fragment ion possesses a lower intensity (i.e. 1.6-fold signal loss). To overcome this issue, the separation of the two isomeric metabolites has to be considered. By implementing the DMS interface on the mass analyzer, approximately a 2-fold signal loss is expected compared to the standard nanoflow interface.¹⁴⁷ But by removing chemical interferences, DMS allows for improving the signal-to-noise ratios and thus lowers the limits of detection and quantification.

Another example is glucuronide metabolites that may experience fragmentation in the atmosphere-to-vacuum interface, which subsequently leads to the formation of the parent drug. Without separation before the MS detection, the differentiation between the products

of degradation from the actual parent drug is not possible and leads to an over-estimation of the latter. This is the case for morphine (MOR), which is metabolized in the inactive metabolite morphine-3-glucuronide (M3G) and to a lesser extent to the pharmacologically active compound morphine-6-glucuronide (M6G). Finally, the metabolism of tramadol (TRMD) leads to the formation of two isomeric metabolites: O-desmethyl-tramadol (ODT) and N-desmethyl-tramadol (NDT) after O- and N-demethylation, respectively. These metabolites are required to be resolved to increase the confidence of TRMD intake determination.

The following discussion focuses on the separation of the three pairs of isomeric metabolites mentioned above and the quality of the separation is considered, considering the resolution (R_s) between the two peaks (Table IV-3), calculated according to Equation 2.

Table IV- 3. Summary of the resolution obtained for the three pairs of isomeric metabolites separated by DMS with or without organic modifiers or by LC-SRM/MS

	BZE/NCOC	ODT/NDT	MOR/MxG	M3G/M6G
No modifier	0.2	0.6	0.4	0.0
ACN	2.9	4.0	2.3	0.0
IPA	0.3	0.8	1.4	0.0
Acetone	2.5	6.6	3.0	0.0
EtOH	1.1	4.6	3.2	1.0
MeOH	0.5	4.3	2.9	1.1
LC-MS	2.3	14.7	3.7* / 1.4**	4.7

* R_s . between MOR and M3G / ** R_s . between MOR and M6G

a) In-solution separation of a mixture of drugs of abuse

Performance of gas-phase separation by modifier-assisted differential ion mobility spectrometry is compared to reversed-phase liquid chromatography commonly (RPLC) used in routine analysis, where elution order can be better predicted according to the analytes polarity. For that purpose, an RPLC separation was developed to separate the set of 30 DoA within 8.5 minutes. Since their calculated $\log P/\log D(\text{pH}=3)$ values are quite different (Table IV-4), the pairs of isomeric metabolites are easily separated by LC with resolution values higher than 1.5: i) BZE and NCOC are separated with $R_s = 2.7$ (Fig. S-4.a); ii) ODT and NDT with $R_s = 14.7$ (Fig. S-4.b); iii) despite their high polarity M3G and M6G could be separated with an R_s of 4.7 (Fig. S-4.c), and are separated from their parent compound MOR.

Table IV- 4. Calculated* logP/logD_(pH=3) for the three pairs of isomeric metabolites and for morphine.

	logP	logD _(pH=3)
BZE	2.26	-0.46
NCOC	3.11	0.01
ODT	1.78	-1.32
NDT	1.68	-1.42
M3G	-1.56	-4.16
M6G	0.69	-1.92
MOR	0.87	-2.22

* Calculated with ACD/Labs software suite (v.11.02, Toronto, Canada)

b) Gas-phase separation of isomeric metabolites

Previous data showed that BZE and NCOC are easily separated by RPLC-MS. To achieve their separation by DMS in the gas-phase, the addition of a polar organic modifier in the drift gas is required. With ACN (Fig. IV- 13.f) and acetone (Fig. IV- 13.g), BZE and NCOC are successfully resolved ($R_s = 3$) while with alcohols like IPA, MeOH (Fig. IV- 13.h) or EtOH, the resolution remains insufficient ($R_s < 1$). Similarly, tramadol isomeric metabolites, i.e. ODT and NDT, are separated by DMS with the addition of ACN (Fig. IV- 13.f), acetone (Fig. IV- 13.g), EtOH and MeOH (Fig. IV- 13.h) with R_s values above 4. In contrast, the best DMS separation of morphine glucuronides (M3G/M6G) is achieved with MeOH (Fig. IV- 13.h) with a $R_s=0.9$ that is not sufficient to resolve peaks at the baseline. All the other evaluated modifiers did not succeed in their separation. However, morphine can be separated from its two glucuronides by DMS, that prevents a probable over-estimation of its MS response.

The analyte-organic modifier clustering/declustering phenomenon occurring in the low- and high-field portion during DMS separation is still not well characterized. Therefore, compensation voltages are difficult to predict in contrast to RPLC where elution order can be estimated based on the analyte's polarity. As a matter of fact, no correlation between retention times (LC) and compensation voltages (DMS with or without modifier) is observed (Fig. S-5). This illustrates nicely the orthogonality of these two techniques for low molecular weight compounds analysis where hyphenation of both techniques has shown increased peak capacity and improved data quality.¹⁴⁸

4.3.3.3. Hyphenation of DMS-MS/MS with liquid extraction-based surface sampling

a) Analytical platform

While several steps including sample homogenization are required prior to LC-MS or GC-MS analyses, sample preparation needed for the direct surface analyses only consists of cutting tissue sections (generally from 10 to 20 μm ; knowing that most eukaryotic animal cells are of 10 - 30 μm) and in thaw-mounting those onto adequate support. In our case stainless steel plates were used, but it could also be glass slides employed for microscopy. Liquid

extraction-based surface analysis (LESA) is then performed with the chip-based nanoelectrospray (nES) TriVersa NanoMate platform (Fig. IV- 12) as previously described by Kertesz *et al.*¹⁴⁹⁻¹⁵¹ A LESA cycle consists of aspirating the extractive solvent and dispensing a droplet on top of the tissue section to form a liquid micro-junction for a given extraction time (e.g. typically 10 seconds). Then, the solution is sucked back into the pipette tip and the sample extract is infused through the integrated nES microchip by applying a high voltage to the conductive pipette tip with a small gas (nitrogen) back-pressure to the solution. Considering that a new pipette tip and a new nozzle are used for each analysis, any sample-to-sample carry-over is avoided.

After surface sampling, the analytes are separated by DMS as previously described prior to their sampling in the mass spectrometer. At this stage both qualitative (QUAL) and quantitative (QUAN) information can be acquired. For that purpose, it is possible to operate the compensation voltage either in scanning mode or at a fixed value. In CoV scanning mode, chromatographic-like peaks are obtained, which allows one to monitor the separation, in particular of the isomeric species, and to determine the CoV value (i.e. at the peak apex) of each analyte for their optimal transmission. QUAL data (i.e. tissue profiling) are also recorded in the data-dependent acquisition (DDA) mode to confirm the identity of the analytes detected in the tissue section. The DMS separation of the 30 drugs of abuse can be achieved in less than 5 minutes, which is compatible with the duration of a stable spray (i.e. 20-25 minutes) delivered by the chip-based nanoelectrospray device. QUAN data are recorded in the selected reaction monitoring (SRM) acquisition mode at a fixed optimized CoV value for each DoA. The CoV can be optimized for each analyte due to the low variation from one analysis to another (i.e. RSD < 5% with ACN as modifier, Table IV- 2). This setup allows for a total analysis time below 1.5 minutes per samples, i.e. from the surface sampling to the infusion and finally the acquisition of the SRM signal over a period of 30 seconds. This considerably increases the overall analysis throughput for quantification, compared to conventional LC-MS or GC-MS assays, which require analysis times within the range of 5-10 minutes.

b) Choice of the solvent for the liquid extraction and extraction efficiency

The following criteria have to be considered in the selection of the extractive solvent composition: i) a high organic content to extract low molecular weight compounds (i.e. solvent typically used in routine methods to extract drugs from tissue homogenate), ii) a stable liquid micro-junction in-between the pipette tip and the tissue section and iii) a stable nanoelectrospray.

In our experiment, the extractive solvent consists of 75% MeOH in water with 0.1% of formic acid. With higher organic content (i.e. up to 80%), the liquid micro-junction between the pipette tip and the tissue section is still stable but the nES becomes unstable with this

type of chip. Although MeOH is a good solvent to extract small drugs, it also limits the dispersion across the surface compared to other organic solvents such as IPA or ACN.¹⁵²

After one LESA cycle, 75% of the total amount of standards spotted (i.e. 250 ng) onto a stainless steel plate could be re-solubilized and infused into the MS analyzer. After five successive extractions from the same location, the signal measured is less than 1% of its original value (results not shown). From these results we can expect that the extraction from tissue would not be complete, which correlates with previously published data.¹⁵² Nevertheless, the extraction process is reproducible, which is one of the most important criteria to fulfill in order to perform quantitative analysis. Also, partial extraction enables additional sampling of the same area to perform complementary analysis.

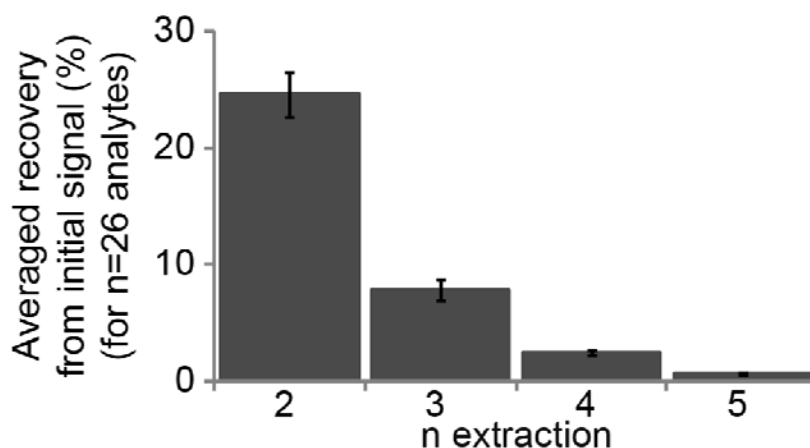


Figure IV- 15. Recovery after 5 successive extractions of 250 ng of standards from the same location (n=3). Error bars display +/- confidence interval at 95%. After 5 extractions, the signal measured is less than 1% of the original signal. 75% of the total amount extracted after 1 LESA cycle.

c) Application of LESA-DMS-MS/MS to the investigation of drugs of abuse in postmortem human tissue sections

The screening of the different DoA was performed on LESA extracts from kidney and muscle samples from different subjects. Considering the separation, selectivity and sensitivity parameters and previous investigations, ACN was selected as DMS modifier for investigating these tissue samples. The SRM survey monitoring of human kidney (Fig. IV- 16.a) reveals the presence of cocaine (CoV = -28.8 V) and two of its metabolites: EME (CoV = -48.0) and BZE (CoV = -31.4 V), as well as methadone (CoV = -17.1 V) and midazolam (CoV = -8.5 V). The identity of these compounds has been confirmed by the triggered MS/MS spectra at the corresponding CoV, as shown for BZE in this sample (Fig. IV- 16.b). MALDI-MS imaging experiments have been performed on the same tissue section and the results obtained correlate those obtained with the LESA-DMS-MS/MS platform.¹⁵³ The analysis of the human muscle section revealed the presence of olanzapine (CoV = -18.8 V) and promazine (CoV = -28.3 V) (Fig. IV- 16.c). These same analytes were also found in the human kidney sections from the same individual. These results correlate with the qualitative screening achieved by LC-MS (Table IV- 5).

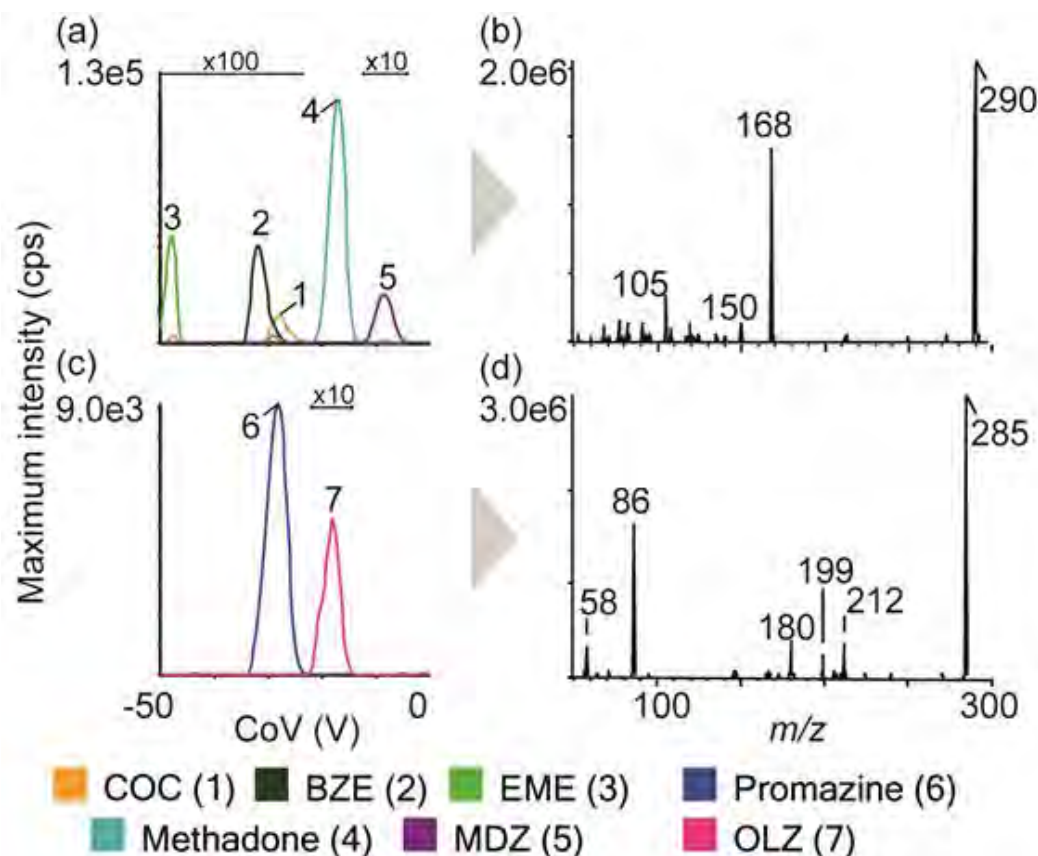


Figure IV- 16. Drugs of abuse screening by LESA-DMS-MS/MS in postmortem tissue sections of human kidney or muscle from two different individuals. CoV ramp survey SRM traces of (a) COC, BZE, EME, methadone and midazolam found in kidney tissue, and (c) promazine and olanzapine found in muscle tissue. Representative confirmatory MS/MS spectrum for (b) BZE and (d) promazine. SV = 4.0 kV with 1.5%ACN as organic modifier.

Table IV- 5. Targeted screening of analytes in postmortem human kidney and muscle samples by LC-MS and LESA-DMS-MS. ACN is used as organic modifier for the DMS separation. n.a. = not available. n.d. = signal lower than the limit of detection (i.e. < S/N=3)
a) Only traces of norDZ (i.e S/N < 5) were detected in kidney sample for Individual B but the sensibility was not sufficient for its structural confirmation.

Individual ID	Type of tissue	List of analytes detected by LC-MS	List of analytes detected by LESA-DMS-MS
Individual A	Kidney	Methadone	Methadone
		Midazolam	Midazolam
		COC	COC
		BZE	BZE
		EME	EME
Individual B	Kidney	Promazine	Promazine
		Olanzapine	Olanzapine
		OLZ metabolite	n.d.
		norDZ	norDZ ^{a)}
	Muscle	n.a.	Promazine Olanzapine

d) Assessment of the relevance of the LESA-MA-DMS-SRM/MS platform for absolute quantification

After compound identification the ultimate goal in analytical chemistry is the absolute quantification of the analyte, but the preparation of adequate calibration standards in tissues and the addition of an internal standard remains challenging. So far absolute quantification has mainly been investigated with a MALDI approach.¹⁵⁴ Quantification curves can be built by defining a region of interest where a known amount of analyte is spotted. The spot area, density and thickness of the tissue can then be defined and the results are expressed as amount (mol or g), amount per unit area, or amount per mass tissue.

In the present work to evaluate linearity, precision, and accuracy, calibration curves were built for cocaine and its metabolites by spiking 1 μ L of calibration standard solution on the surface of blank tissue sections. Then, 1 μ L of a solution of COC-d3, used as internal standard, is subsequently spotted on the top of target and tissues samples spots, so that the spot area is the same for the analytes and the internal standard on the tissue section. The system was found to be linear over three orders of magnitude (2 to 1'000 ng/mL), with acceptable precision (0.2-11 %, n=3) and accuracy (95.9% - 108%, n=3) (Table IV-6). Precision and accuracy for QC samples at five different concentration levels were found to be in the range 0.2-11 % and 96-110%, respectively.

Intra- and inter- section reproducibility has been determined by analyzing cocaine and its metabolites in real forensic tissues samples. Data collected after the extraction from adjacent kidney sections and after normalization with the deuterated standard showed intra- and inter- section reproducibility within the range 0 to 25% (Table IV-7), which is close to the 20% of variation reported in the literature with other techniques.¹⁵⁵

Table IV- 6. Quantitative LESA-DMS-SRM/MS: precision and accuracy calculated for cocaine. COC-d3 was added in the CAL and QC samples at a final concentration of 250 ng/mL and spotted onto tissue sections. The extraction was performed with 75% MeOH, 0.1% HCOOH. Concentrations are given in ng/mL. The accuracy and the precision are calculated for n=3 replicates.

Sample	Expected concentration	Calculated concentration			Mean	Accuracy	RSD
		n=1	n=2	n=3			
CAL1	4.76	4.51	4.68	4.50	4.57	95.9%	2.3%
CAL2	9.52	10.57	9.82	10.58	10.32	108.4%	4.2%
CAL3	95.24	92.29	96.02	94.71	94.34	99.1%	2.0%
CAL4	238.10	233.39	235.86	220.04	229.76	96.5%	3.7%
CAL5	476.19	450.70	459.34	462.79	457.61	96.1%	1.4%
CAL6	952.38	997.06	972.11	1002.16	990.45	104.0%	1.6%
QC1	4.76	4.83	4.99	5.90	5.24	110.1%	11.0%
QC2	9.52	9.91	9.53	10.00	9.81	103.1%	2.5%
QC3	95.24	94.46	94.13	94.22	94.27	99.0%	0.2%
QC4	238.10	201.98	240.97	243.13	228.69	96.0%	10.1%
QC5	476.19	478.11	480.21	483.78	480.70	100.9%	0.6%
r ²		0.99469	0.99922	0.99391			
Slope		0.83883	0.83211	0.82849			
Intercept		0.00214	0.00268	0.00135			

Table IV- 7. Intra- and inter- tissue sections reproducibility after extraction of cocaine and its isomeric metabolites BZE and NCOC detected from different locations on 3 adjacent kidney sections.

	COC	BZE	NCOC
RSD _{intra} (s1) n=4	9%	24%	20%
RSD _{intra} (s2) n=4	23%	15%	12%
RSD _{intra} (s3) n=4	14%	15%	11%
RSD _{inter} n=12	26%	24%	19%

The addition of a DMS separation in the analytical platform allows a reduction of noise by eliminating interferences and therefore lowers the limits of detection (LOD) and of quantification (LOQ). The LOD and LOQ were estimated from the absolute signal measured by LESA-DMS-SRM/MS for cocaine and its metabolite BZE detected in the Individual A (Figure IV- 16) from four different extractions (i.e. two spots analyzed from two different tissue sections). As the noise measured was close to zero, an arbitrary value of 10 cps was set for signal-to-noise (S/N) ratios calculation. The S/N were calculated and then correlated to the actual concentrations determined by LC-MS from tissue homogenates (i.e. 73 ng/g for cocaine and 198 ng/g for BZE; Table IV-8). Assuming a theoretical S/N for the LOD is of *ca.* 3

and a S/N for the LOQ is of *ca.* 10, the LOD and the LOQ were found to be 10 and 30 ng/g, respectively, for cocaine. For benzoylecgonine, the LOD and the LOQ were found to be 5 and 20 ng/g, respectively. The sensitivity obtained with the new analytical platform is sufficient for toxicological screening of various drugs of abuse (e.g. cocaine, opiates and opioids, amphetamines) in forensic postmortem samples including human kidneys, of which concentrations are mainly distributed within the range of 70 to 1'540 ng per g of tissue (Table IV-8). As a typical comparison, a validated GC-MS/MS method to study cocaine metabolism in human primary cultured renal cells could measure down to 3 ng/g.¹⁵⁵ Another study reports cases where 20 to 100 ng/mL of cocaine have been quantified from liver and brain homogenates.¹⁵⁶ With MALDI-MS imaging using both ion-trap and quadrupole-time-of-flight (QqTOF) instrumental platforms operated in MS/MS mode with a wide precursor ion isolation window, the lowest quantity which could be measured in human brain was 50 ng/g.¹⁵⁷⁻¹⁵⁸

Table IV-8. Quantitative analysis of selected analytes by LC-MS in postmortem human kidney samples after homogenization and extraction. *a) EME and midazolam were not included in the quantitative analysis by LC-MS. b) ULOQ = upper limit of quantification (=250 ng/g).*

Sample ID	Analytes	Concentration (ng/g, n=5)
Individual A ^{a)}	Methadone	> ULOQ ^{b)}
	COC	73 ± 12
	BZE	198 ± 28
Individual B	Promazine	1'539 ± 1
	Olanzapine	414.60 ± 0.04
	OLZ metabolite	73.20 ± 0.03
	NorDZ	869 ± 1

Direct surface analysis in general has to deal with an important matrix effect specific to each type of tissue.¹⁵⁹ Because the ion mobility separation occurs directly after the ionization process, it does not prevent ionization suppression effects typically observed in sample extracts from biological samples.¹⁶⁰ Classical approaches to estimate the absolute matrix effects cannot be applied to tissues, therefore extraction efficiency and matrix effects are estimated by the ratio between the signal given by a standard spotted on a stainless steel plate with that of the signal given from the same standard spotted on the tissue section. For cocaine, amphetamines, and opioids, the MS signal decreases significantly depending on the tissue and was found to be typically in the range of 9-12 times for kidneys and 40-100 times for muscles (Fig. S-6). Results may be biased due to a different wetting of the sample between a stainless steel plate and a tissue sample and needs to be further investigated. However, the relatively low process efficiency does not adversely affect the quantification performance of the method.

4.3.4. Conclusion and perspectives

A rapid, selective and sensitive approach combining liquid extraction surface analysis and modifier-assisted differential ion mobility spectrometry mass spectrometry is described as a powerful QUAL/QUAN analysis platform to investigate drugs of abuse and metabolites in postmortem forensic samples, down to the ng of analyte per g of tissue. By removing interferences, DMS allows increasing the selectivity and improving the signal-to-noise ratio to achieve lower limits of quantification in the range of ng per g of tissue. Although the limits of detection and quantification are higher for the LESA-DMS-SRM/MS compared to those reached with LC and/or GC-SRM/MS (i.e. at least 10-fold), this is sufficient to perform a complete toxicological screening of drugs of abuse in postmortem tissues. Although the differential mobility behavior of analytes cannot be clearly anticipated, DMS can act as a chromatographic separation of ions in the gas-phase and is demonstrated to considerably improve the selectivity of a conventional surface sampling-based platform operated at atmospheric pressure prior to MS detection. The addition of a polar organic modifier in the drift gas is mandatory to improve the peak capacity and the separation power of DMS. Different modifiers have been assessed, and results indicate that different selectivity is provided in function of the nature of the modifier, i.e. alcohols such as MeOH/EtOH/IPA against ACN/acetone. As a result, isomeric metabolites such as hydroxylated or demethylated ones are resolved, and glucuronide metabolites are differentiated to the parent drug, which prevent the overestimation of the latter in case of in-source fragmentation. This work is also a first step toward the use of the LESA-DMS-SRM/MS platform for absolute quantification directly from tissue sections. We demonstrate herein the suitability of the platform for QUAN purpose in terms of extraction reproducibility (i.e. variation less than 25%) as well as for the linearity of the response over three orders of magnitude. The challenging part of this work remains the generation of reference standards representative of real samples to generate relevant calibration curves. The application of LESA-DMS-MS for the analysis of low molecular weight compounds such as drugs of abuse and their metabolites from tissue sections demonstrates the great potential of gas-phase separation techniques as an alternative to liquid-phase chromatography and may be very interesting for compounds that are difficult to analyze by LC such as oligonucleotides or amino acids. The platform could also be used as a powerful tool for metabolite profiling within the scope of early drug metabolism study, together with other imaging approaches like whole body autoradiography, MALDI-MS or TOF-SIMS imaging, which offer higher resolution capabilities.

4.3.5. Acknowledgments

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4.3.6. Supporting Information

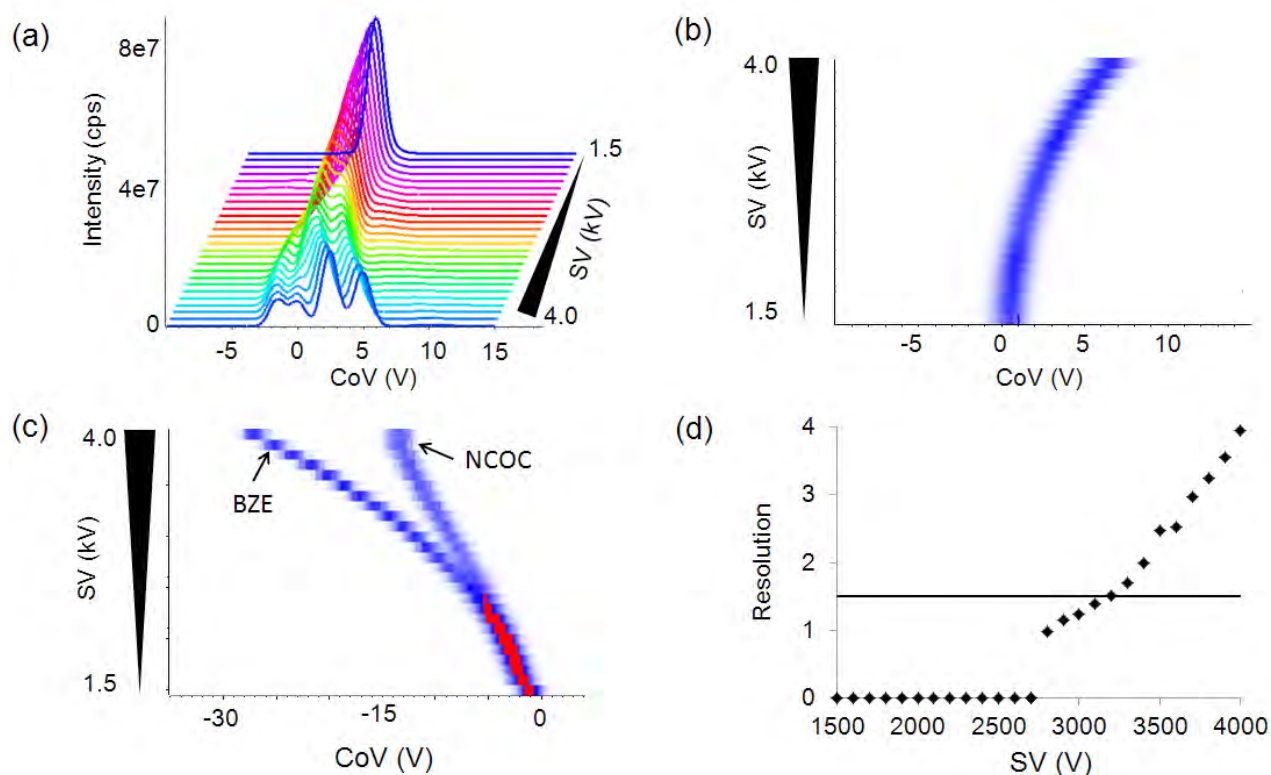


Figure S-1. Effect of the separation voltage and of the addition of ACN in the drift gas on the DMS separation.

(a) Offset TIC traces from the DMS separation of five amphetamines at different SV amplitudes without modifier. Heat maps based on the XIC SRM trace m/z 290 > m/z 168 monitored for BZE/NCOC **(b)** without modifier and **(c)** with ACN added into the drift gas. **(d)** Resolution calculated for the two isomers BZE and NCOC at different SV values with ACN as modifier.

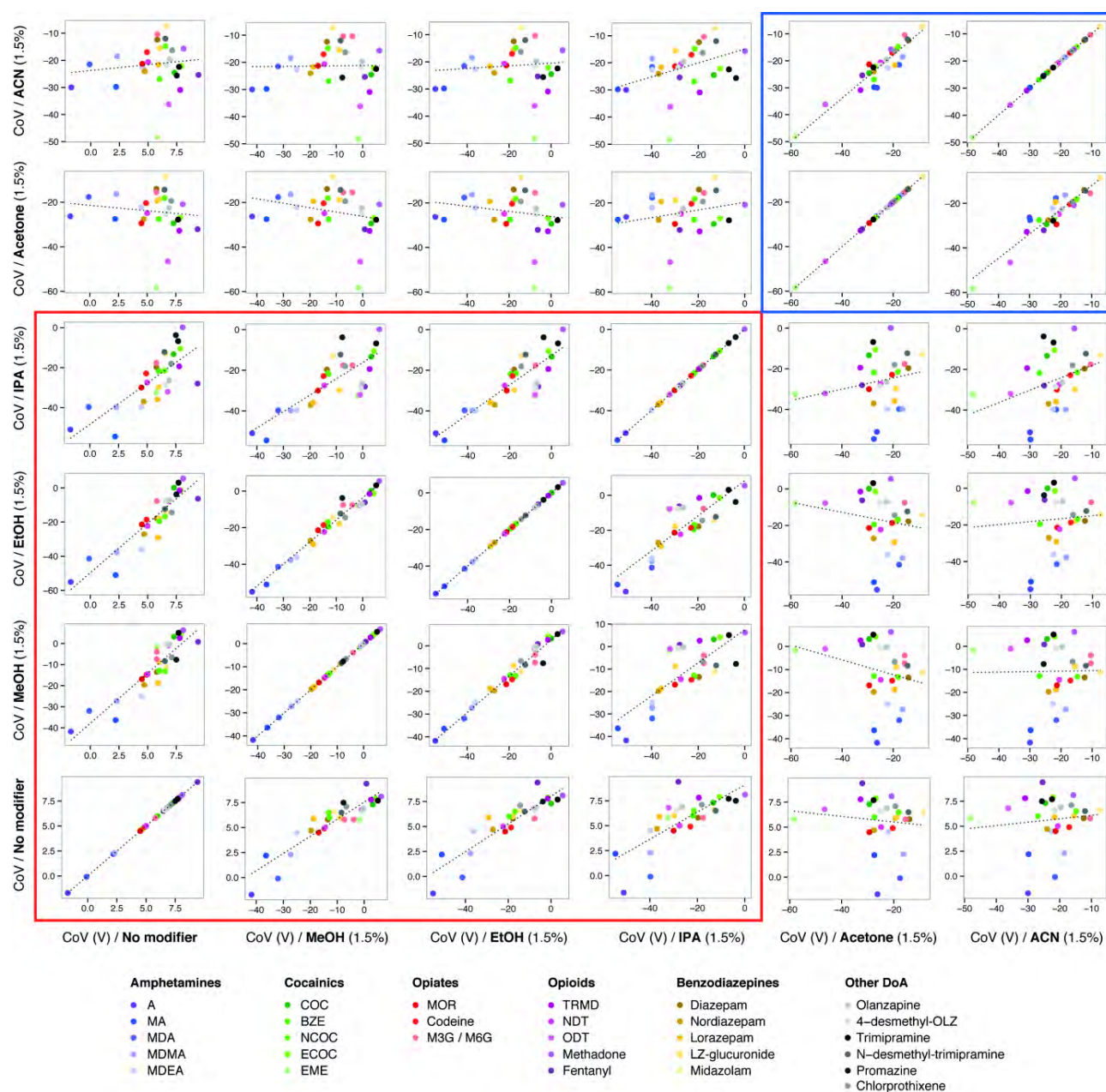


Figure S-2. Pairwise comparison of different organic modifiers for the DMS separation of a set of DoA. CoV values of each analyte plotted for two modifier conditions at a time. The squared boxes indicate good correlation between alcohols modifiers or nitrogen only (red box) and between ACN and acetone modifiers (blue box).

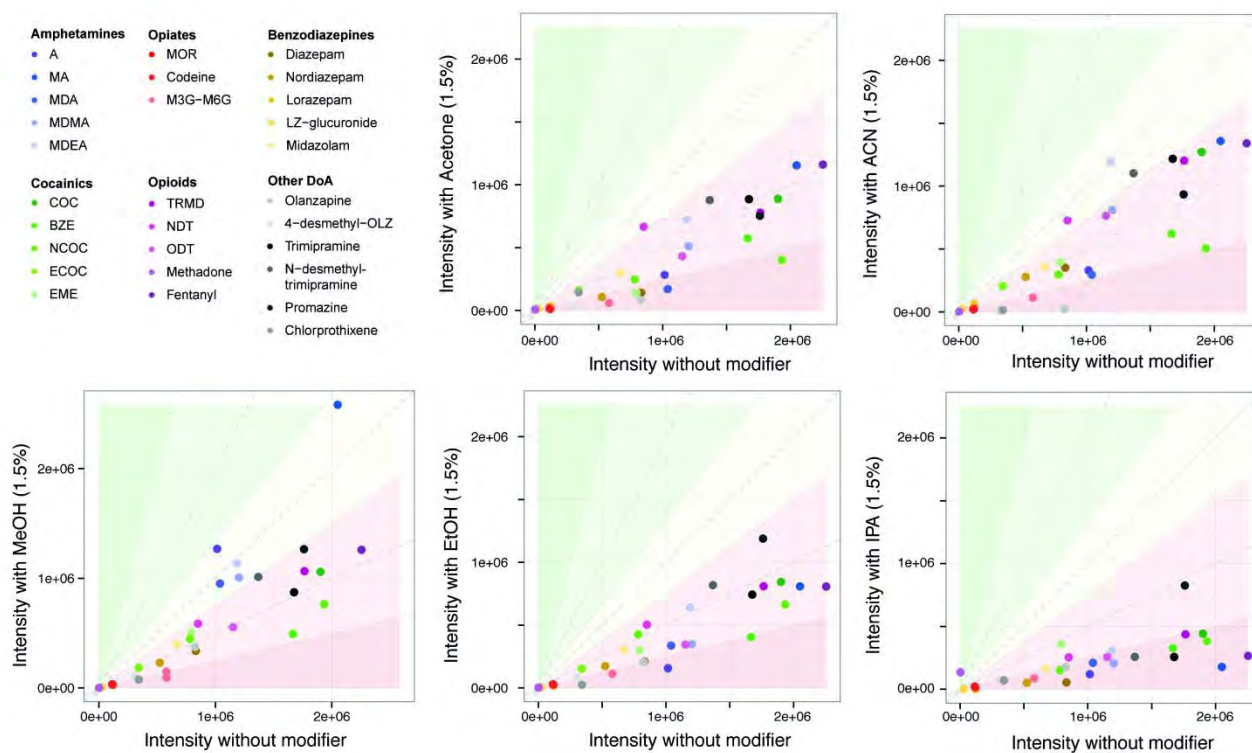


Figure S-3. Influence of the organic modifiers on the absolute MS response of each analyte. The shaded zones are colored relatively to the intensity measured without organic modifier (reference): dark red = < 25%, light red = 25-75%, yellow = 75-125%, light green = 125-175% and dark green = > 175%.

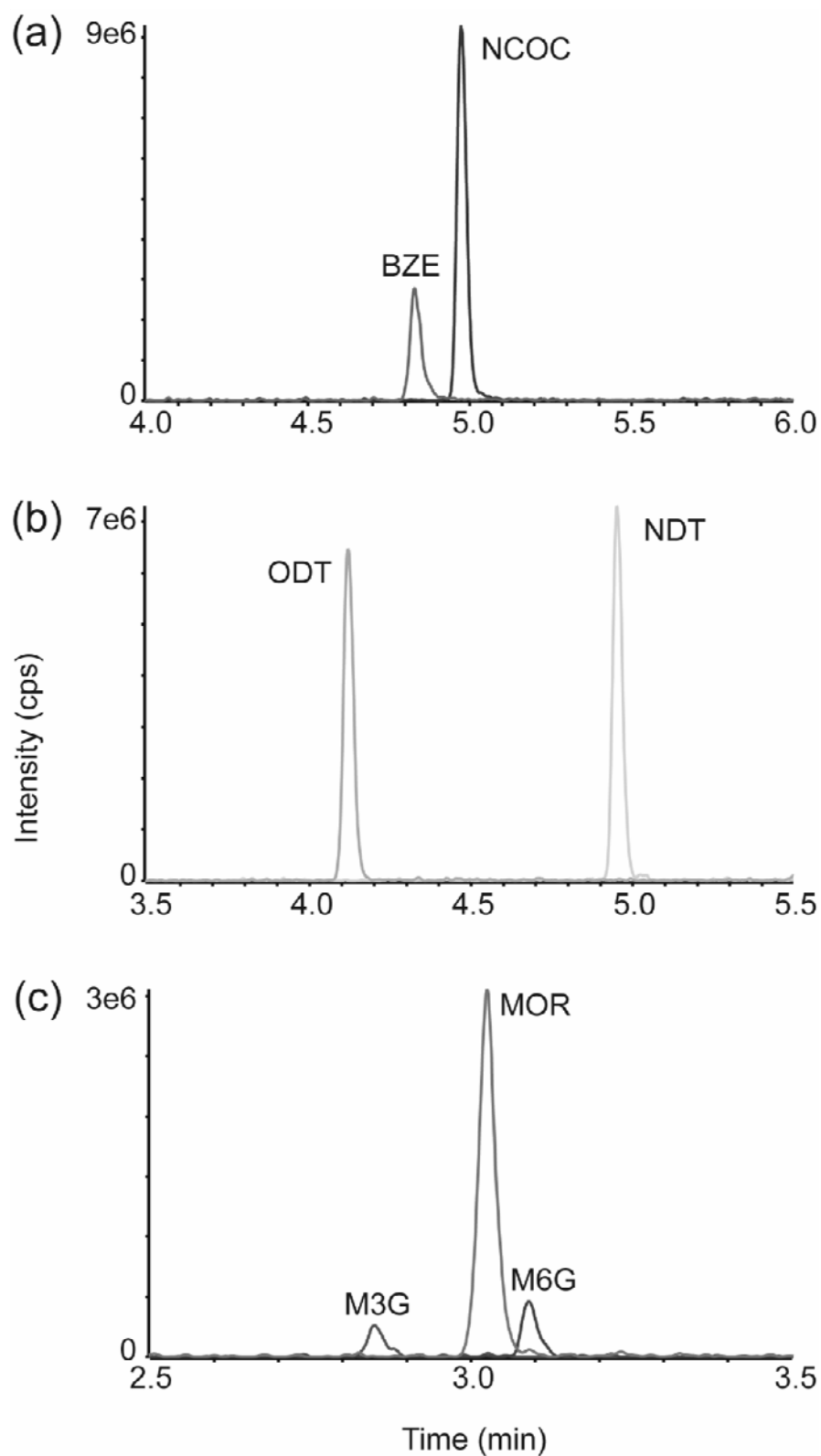


Figure S-4. Separation of isomeric metabolites by RPLC-MS. Extracted ion chromatograms for (a) BZE and NCOC; (b) ODT and NDT (TRMD metabolites); and (c) morphine (MOR), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G).

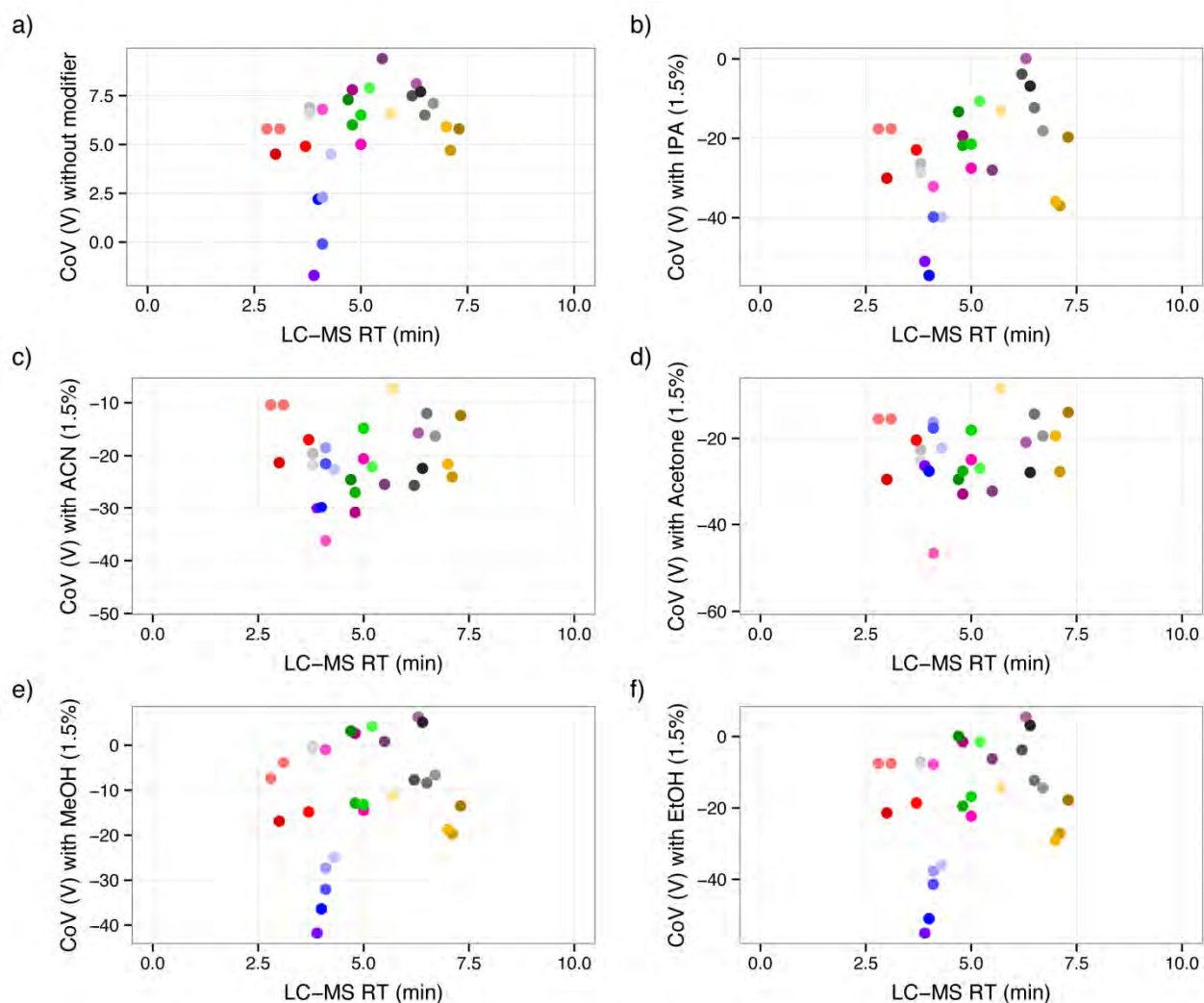


Figure S-5. Comparison between in-solution (LC) and gas-phase (DMS) separations. CoV values obtained for the 30 analytes of a 100 ng/mL mixture using the organic modifiers (a) no modifier; (b) IPA; (c) ACN; (d) acetone; (e) MeOH and (f) EtOH, plotted against the retention time obtained for these analytes after their separation by liquid chromatography. Spots colors are as in Figure S-2 or Figure S-3.

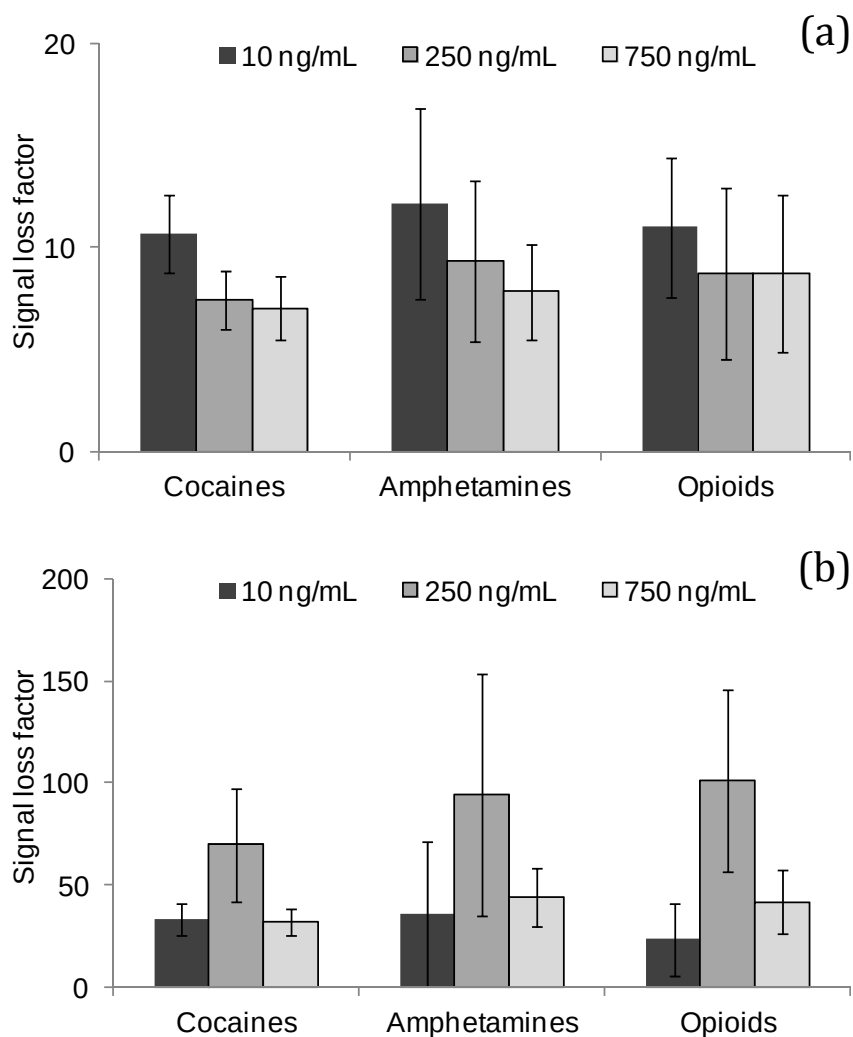


Figure S-6. Signal loss factors calculated by comparing the MS intensity of standard solutions (i.e. 10, 250, 750 ng/ml) spotted on stainless steel plate with the signal of those solutions spotted on postmortem human (a) kidney and (b) muscle sections. The results express the average value for all the analytes of three different classes: cocaines, amphetamines and opioids. Error bars represent $\pm$ one standard deviation.

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**CHAPTER 5. Quantitative MS Imaging –
*Experimental considerations to generate
SRM-based images with good quality and
suitable standards for direct
quantification from tissue sections***

CHAPTER 5.

Quantitative MS Imaging (QMSI)

Part 1 – Challenges for quantitative mass spectrometry imaging



“The greater the obstacle, the more glory in overcoming it”

Molière (1622 - 1673)

5.1.1. Introduction

Mass spectrometry imaging (MSI) has been proven as a reliable technique for mapping diverse classes of compounds within biological samples. One of the strengths of MS-based imaging techniques is the ability to distinguish between parent drugs and their metabolites (without requiring labeling) and other endogenous molecules. In addition, the mass spectrometry capability allows for the discovery of unknowns. The characterization of the variability from one sample to another is of prime importance in linking to biological relevance and the development of a robust and accurate approach is necessary. In this respect, the ultimate goal of molecular imaging techniques based on mass spectrometry would be to correlate the exact amount of a given analyte with its specific localization, within a single experiment.

For that purpose, QWBA is the only imaging technique so far established as quantitative and is widely used in drug discovery and development. QWBA is often used in combination to MSI to link the amount of molecules with their localization within the tissue, as long as a radiolabeled compound is available. MSI experiments have also been conducted in parallel to CE-MS² or LC-MS/MS³⁻⁷ as reference, after organ dissection and homogenization. Koeninger *et al.* report a quantification method to analyse olanzapine after its administration to rats at different concentrations. In this study, the integrated MALDI-MSI response of olanzapine was correlated to LC-MS/MS performed on adjacent tissue sections.⁶ However, these techniques are quite expensive (i.e. when using radiolabeled compound), time consuming, and lead to the alteration of the physical integrity of the sample.

Although MS imaging is now well established as a qualitative tool, its use as a quantitative tool is still controversial and absolute quantification from tissue remains challenging. There is an urgent need to prove the quantitative potential of MS imaging, at least demonstrating the robustness of any analytical approach based on MSI. In other words, quantitative information is required to estimate the performance of any analytical platform such as the limits of detection/quantification. The objective is to guarantee the signal detected really comes from the analyte and is not an artifact or interfering ion from the matrix (when used) or from endogenous compounds present in the biological tissue (i.e. when MS and/or MS/MS detection is not selective enough).

If the quality and relevance of quantitative results begins with the careful handling of samples (i.e. during sampling, storage, sample preparation), to prevent sample degradation as much as possible (see Chapter 1, Part 1), the MSI researchers still must address the following challenging questions: how should the ion signal recorded be presented? Does the recorded ion signal display accurately represent the actual amount of a given compound in the tissue? Though the number of publications addressing these issues is increasing, still no single of the methods described has been universally recognized. The objective of this section is to depict the main challenges encountered in quantitative mass spectrometry imaging (QMSI) and discuss the methodologies applied so far.

5.1.2. Challenges and strategies developed in QMSI

5.1.2.1. Tissue suppression effect

To perform absolute quantification directly from tissue sections, several fundamental aspects inherent to the analysis of tissue must be considered. The first challenge is the **ion or tissue suppression effect**. This effect is encountered when an analyte is considered in its chemical and biological environment. The analyte thus experiences ionization competition with endogenous species (see analyte suppression effect for MALDI, Chapter 3, Part 1). An example of these endogenous species is the highly abundant phospholipids present in cell membranes. In some cases, intense lipid signalling may mask the presence of other molecules, including the analyte. Obviously, this ion suppression effect is tissue specific. Then, when the analyte is trapped in tissue, it undergoes many noncovalent interactions and the extraction efficiencies, difficult to characterize, may vary in function of the tissue. As a consequence, the absolute signal intensity is not representative of a targeted analyte's concentration and quantification has to be done taking into account the tissue suppression effect.

5.1.2.2. Quantification by MSI: the quest for the perfect matrix-matched standards

The second challenge is the generation of a suitable dilution series to represent accurately the sample while taking into account the tissue suppression effect. Several attempts based on in-solution, on-tissue and in-tissue strategies have been made and are reviewed here.⁸

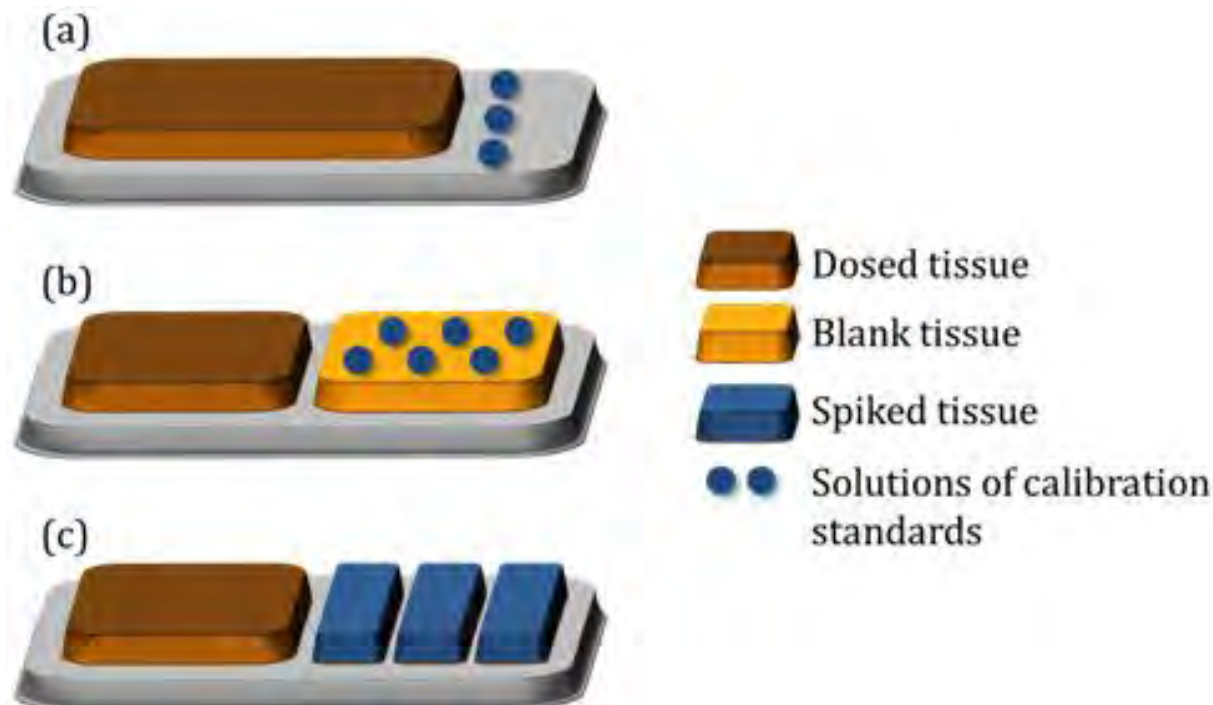


Figure V- 1. Overview of the three different strategies based on (a) in-solution, (b) on-tissue and (c) in-tissue dilution range developed to provide quantitative information using MSI approaches. Reproduced from ref.⁸

a) In-solution dilution range

The simplest method to determine the concentration of an analyte is to use an in-solution dilution range (Figure V- 1.a). This technique does not take into account the tissue suppression effect or the differences in analyte extraction efficiency from complex biomatrices. It can give an approximation of the tissue extinction coefficient (i.e. by comparison between the signal given by the analyte deposited onto the sample plate versus the signal given by the same analyte deposited on the tissue section), but does not accurately represent the actual situation for a given compound trapped in its chemical/biological environment. The “tissue extinction coefficient” (TEC) approach makes partial use of this strategy and is detailed in section §5.1.3.a.).

b) On-tissue strategies

The goal of the “on-tissue” approaches is to introduce the tissue suppression effect in the calibration curve. In 2010, Nilsson *et al.* provided the first evidence that compounds administered by inhaled delivery at standard pharmacological dosage can be quantitatively detected by MALDI-MSI with accuracy and precision, using both MS and MS/MS modes.⁹ The strategy consists of the application of different concentrations (20 fmol-10 pmol) of drug standards to the control tissue, and sections from the control and the dosed tissue sections are coated with matrix at the same time in the automated spraying device. Regions of interest are then drawn and the average intensities from the drug spiked regions are used to build standard curves for both MS and MS/MS experiments, respectively. However, this approach does not take into account the droplet dispersion on the tissue and does not reflect the actual concentration of analyte per gram of tissue, as the signal recorded at each level of concentration is then plotted *versus* the amount spotted (expressed in mol or g).

As mentioned in the introduction, Koeninger *et al.* have reported a quantification method where the integrated MALDI-MSI response correlates LC-MS/MS analysis performed on adjacent tissue sections, after harvesting from rats several single doses of olanzapine at different concentrations.⁶ This demonstrates the linear response of the analyte as a function of the initial dose administered, but again, the method does not allow direct expression of the final result in amount of analyte per mass tissue. Goodwin *et al.* also employed MALDI-MSI to map commonly employed PET ligands in rat brain tissue sections.¹⁰ Compound identification and distribution were confirmed through mass accuracy and MS/MS fragmentation data. Semi-quantitative data is obtained by MALDI MSI analysis via spotting known amounts of the target compound onto the surface of control tissue (final result expressed in mM) to measure both dose-dependent and a time course of distribution and abundance. In all cases, the results have been correlated to LC-MS/MS to demonstrate the robustness of the methods. Also, no internal standard has been used. The normalization of the signal in MS mode used the total ion count (see §5.1.4.b), while no normalization is used in the MS/MS experiments. Finally, although this approach is fast, it is not adapted to the simultaneous analysis of multiple tissues with different tissue suppression effects and can be performed on large organs only (i.e. sufficient space to spot standard droplets).

c) Reconstitution of tissue sections from homogenates spiked with the analyte (in-tissue dilution range)

Because the main challenge in MSI is the generation of standards that reflect the behavior of a compound in its complex biological environment, the third approach suggests generating standards from tissue homogenates, spiked with a known amount of drug. This approach, also called the “matrix-matched standard” method, is widely used and described as the most practical means for producing quantitative images of trace metal distribution in tissue sections by LA-ICP-MS.^{1, 11-14}

Recently, Hare *et al.* have published a protocol for the production of matrix-matched brain tissue standards for imaging by LA-ICP-MS.¹ This protocol consists of homogenization of tissue spiked with the analyte of interest, then reconstituted by fast freezing, sectioned and mounted onto a target plate for further analysis. Calibration curves are then built on the basis of the signal measured from different matrix-matched standards containing increasing concentrations of analytes.

The matrix-matched standard strategy takes into account the ion suppression effect specific to tissue type. Although this approach, like the on-tissue approach, is particularly adapted to single organ drug distribution studies, it holds the advantage of expressing quantitative results in amount of analyte per mass of tissue. Extending the multiple organs study or whole-body tissue section analysis remains time consuming and requires a large amount of tissue material.⁸ Based on this approach, which seems to be promising for QMSI (i.e. Hamm *et al.* report an adequate homogeneity observed with an inter-sections mean signal variation of 15%⁸), we have made first attempts in our lab to establish a protocol for the generation of such in-tissue dilution range for molecular imaging of low molecular compound in postmortem human kidney (Chapter 5, Part 3).

Yet using this approach the best way to represent and normalize the image generated still needs to be discussed. As well as the incorporation of the internal standard in the analytical workflow when using it for targeted approaches:

- a) applying an internal standard on the top of the tissue might not accurately reflect the analyte being extracted from within or beneath the tissue sample, as the internal standard might reside atop the tissue;
- b) applying the internal standard beneath the tissue might best reflect the detected ion signal of the target analyte within the tissue as well as those of calibration standards that have also been applied beneath the tissue. This assumes that the dried standards are “extracted” into the wet tissue when the tissue section is placed over the dried standards.¹⁵

5.1.3. Representation of the MS image: with or without normalization?

Because the ion suppression effect affects the quantitative information generated, it is clearly understandable that raw signal cannot accurately reflect the relative distribution of an analyte undergoing ionization competition with endogenous compounds.

To address this issue, two strategies can be employed: i) the first consists of the characterization and calculation of **tissue-specific suppression effects**; ii) the second utilizes **normalization** of the signal either by an endogenous or exogenous signal, to minimize the outcome of the tissue suppression effect and artifacts which affect the intensity of the mass spectra.

5.1.3.1. Calculation of the ion suppression effects

Due to the inherent specificity of signal suppression in the function of each tissue, but also of the substance of interest, each analyte needs to be calibrated in a separate experiment by spiking each tissue and measuring the resulting MS signal.¹⁶ Stoeckli *et al.* propose to calculate tissue-specific ionization efficiency factors, by simply spraying a solution of the compound standard homogeneously to the surface of the tissue section, and then acquiring MS images.¹⁶ This approach is label-free, which is an advantage for instance in the early stages of discovery where a labeled version of the drug is not available. The tissue-specific ionization efficiency factors are then used to divide the MSI values of the drug in the dosed tissue. This strategy allows the relative quantification to accurately compare the signal for a given analyte from one type of tissue to another. But this does not allow for absolute quantification (i.e. the result is not expressed in gram of analyte per gram of tissue in this case).

A variant of this approach, the so-called “tissue extinction coefficient (TEC)” approach, also makes use of the TEC as a normalization factor and takes into account the ion suppression.⁸ The TEC approach consists of two steps: the first step relies on the determination of tissue- and drug- specific TEC after spraying the compound solution as specified above. A TEC is then calculated for each type of tissue by comparison between the signals from the analyte measured on tissue *versus* on plate. The application of varying concentrations of standard mixed in matrix on serial control rat whole-body tissues demonstrated the non-dependence of the standard concentration on TEC value.¹⁷ Then, a dilution series of analyte is spotted near the dosed tissue section and the matrix solution is sprayed over the entire sample. A calibration curve is then built integrating the TEC and the mean intensity values of the standards.

This allows correlation of the amount of drug per surface unit. The strategy has been successfully applied to the absolute quantification of several drugs such as diazepam, propranolol and olanzapine in dosed mice, and also for endogenous compounds such as insulin and an oncometabolite, either by MALDI-MSI or LESA-MS.¹⁷⁻¹⁸

5.1.3.2. Normalization of the signal

a) Total ion current (TIC) normalization

Several papers report the normalization of all spectra against the total ion current (TIC).^{6, 9-10, 19-20} TIC normalization sums all of the intensities in each spectrum, and a total intensity value is obtained for each spectrum and these totals are then averaged. Each intensity value within a spectrum is then multiplied by the ratio of its total intensity to the average total intensity. It follows that the total ion intensity for each spectrum equals the average total intensity. Although the TIC normalization has been proven to reduce the influence of inhomogeneous matrix coating, which leads to the formation of “matrix hot spots”, there are several limitations to consider for quantitative analysis. For example, if one ion, which is not clinically relevant, gives the most intense signal in one group than in another, the TIC normalization can lead to incorrect suppression of all signal intensities in the group.²¹ In this case, a more robust approach such as normalization by the median intensity of the peaks must be considered.²²

b) Normalization by an internal standard

Utilizing an internal standard for traditional MALDI analysis has been demonstrated to improve quantification, as long as the internal standard and the analyte of interest present similarity in term of ionization efficiencies, structures, physico-chemical properties and sometimes fragmentation pathways.²³⁻²⁵ In QMSI, internal standard normalization aims to compensate for compound-dependent ionization efficiencies, on the condition that the internal standard is homogeneously applied over the entire tissue section. Selected examples are presented in Table V- 1. The normalization can be performed by endogenous signal, from a peptide in the same mass range as the targeted analyte²⁶ or a lipid species, for instance.²⁷ The main limitation of this approach is that the endogenous compound may not be distributed homogeneously over the tissue section.

Normalization using an internal standard can incorporate a labeled version of the analyte^{15, 28-33} or a structural analog.³⁴ In a targeted approach, the use of isotopically labeled internal standards has been proven relevant for the quantification of endogenous compounds (e.g. acetyl-l-carnitine from piglet brain³⁰), and also for exogenous compounds (e.g. cocaine from brain tissue^{15, 28}). The different strategies diverge in methods to apply the internal standard in the MSI workflow, i.e. spotted/sprayed on the top or underneath the tissue section. In their latest publication, Pirman *et al.* report how the use of internal standard helps to account for signal variations resulting in significant differences in analyte extraction, co-crystallization, and ionization across a sample and thus identify tissue-specific signal variation.³³ In the approach, the analyte-to-internal standard ratio is monitored and the differences in ion signal are taken into account, resulting in images that better represent the analyte concentration. These experiments have been performed with several types of tissue. In certain tissues (e.g. liver, kidney), the attenuation of the signal is up to 90%. Nevertheless, monitoring the analyte-to-internal standard ratio is demonstrated to take into account these differences. Källback *et al.* recently introduced a novel mass spectrometry imaging software

assisting labeled normalization and quantification of drugs and neuropeptides directly in tissue sections.³² Different normalization methods were employed. Among them, the labeled normalization displayed the best linear regression fit. These observations further emphasize the need for an internal standard in the MSI workflow, in particular for targeted approach.³²⁻³³

5.1.3.3. Calculation of the final concentration in amount of analyte (mol or g) per gram of tissue with strategies using standard spotted

Some of the publications utilizing standard spotted at a given concentration, either onto or beneath the tissue sections, express the final result in amount of analyte (mol or gram) per gram of tissue.^{15, 17-18, 28, 30} This is calculated from the area of the detected spot in the image (i.e. to generate a concentration per cm²) and then the volume of the tissue within based on the thickness of the tissue section. The grams of the wet weight of tissue are finally determined from this volume, using the estimated density of tissue (usually *ca.* 1.00). It is worth noting that this calculation can only be done assuming that 100% of the analyte is extracted from a given volume of tissue, which could give a good estimation of the limits of the methods. However, it is also true that the actual extraction recovery is difficult to characterize utilizing these approaches.

5.1.4. Conclusions

Quantification by mass spectrometry imaging is certainly a reoccurring topic of the current discussions by experts in the field and collective efforts are put together to demonstrate the quantitative ability of MS-based imaging approaches. Recently, two software programs have been proposed to the MSI researchers community to assist and speed up data treatment in quantification by MSI.^{32, 35}

Different strategies have been developed for QMSI for different applications, and one can find label-free or labeled approaches for the quantification of endogenous or exogenous compounds, from small drugs to peptides or proteins. Most of them have been proven robust in term of linearity and attempts usually correlate well with LC-MS results of tissue homogenates. The quest for the perfect dilution series to better mimic the behavior of an analyte in its biological environment is challenging, and has to consider several fundamental aspects inherent to the analysis of tissue, such as tissue- and analyte- specific suppression effects, analyte extraction efficiency from the tissue sections, and diffusion of the analyte standards within the tissue when used. Most of these approaches fulfill some of these limitations, but rarely all of them.

The importance of signal normalization in taking into account these variations has been highlighted, but here again several approaches are proposed. Strategies employing an internal standard are suitable approach to normalize the MS images. However, the way of incorporation into the MSI analytical workflow is still being discussed.

With respect to promoting MS imaging as quantitative tool, the two following parts of this last Chapter will be articulated around two objectives:

- The first objective is to discuss instrumental parameters and data processing influencing the quality of the MALDI-MS images generated. Part 2 also discusses the use of a deuterated standard to normalize the images in a targeted approach using the selected reaction monitoring mode;
- The second objective is to propose a protocol to generate a standard based on the “in-tissue” dilution range approach. Part 3 presents the preliminary results obtained to generate such standards for the quantification of drugs of abuse (i.e cocaine and one of its metabolite) in postmortem human kidney samples.

Table V- 1. Selected publications using normalization by an internal standard in MSI.

Ion source	Mass analyzer	Commercial instrument	Biological sample	Analyte	Internal standard	Method used for normalization	References
MALDI (1 kHz, 355-nm Nd:YAG laser with a spot diameter of 100x200 µm)	QqQ _{LIT}	Flashquant (AB Sciex)	WB rat section	Novartis compound	-	-	Hopfgartner <i>et al.</i> ³⁰
MALDI (1 kHz, 355-nm Nd:YAG laser with a spot diameter of 100x200 µm)	QqQ _{LIT}	Flashquant (AB Sciex)	Single intact hair samples	Cocaine and its metabolites	Deuterated version of the analytes; Homogenously applied on the top of the tissue section	SRM ; (unit isolation window)	Porta <i>et al.</i> ²³
MALDI (1 kHz, 355-nm Nd:YAG laser with a spot diameter of 100x200 µm)	QqQ _{LIT}	Flashquant (AB Sciex)	Lung	Moxifloxacin (tuberculosis)	Analog (warfarin); Homogenously applied on the top of the tissue section	SRM ; (unit isolation window)	Prideaux <i>et al.</i> ²⁸
MALDI (300 Hz, Nd:YAG laser)	TOF	Voyager SSTR (AB Sciex); 4700 Proteomics Analyzer (AB Sciex)	WBA	Peptides and proteins; Drug imaging	Internal calibrant (peptide in the same mass range than the targeted analyte)	MS; MS/MS	Rohner <i>et al.</i> ²⁰
DESI	LIT	MALDI LTQ (Thermo-Fisher Scientific)	Lung, kidney, testis and brain from rats	Clozapine; N-desmethyl-metabolite	Endogenous lipid specie	Full scan mode	Wiseman <i>et al.</i> ³¹
MALDI (60-Hz, 337-nm nitrogen laser with a spot size of 100 µm)	LIT	MALDI LTQ-XL (Thermo-Fisher Scientific)	Human brain of cocaine users	Cocaine	Deuterated version of the analytes (COC-d3); Homogenously applied beneath the tissue section	MS/MS with a 6-Da wide-isolation window; Scan-to-scan normalization COC-to-COC-d3	Reich <i>et al.</i> ²² ; Pirman <i>et al.</i> ¹³

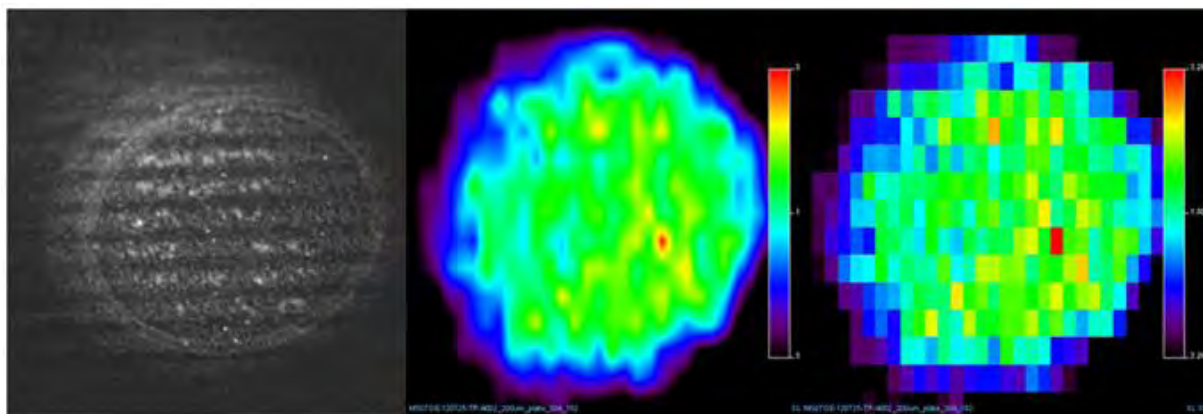
Table V- 1. Continued.

Ion source	Mass analyzer	Commercial instrument	Biological sample	Analyte	Internal standard	Method used for normalization	References
MALDI (200-Hz, 355-nm YAG laser with a spot diameter of 150 μm)	Q-IM-TOF	Synapt MALDI-Q-Ion Mobility (T-wave cell) time-of-flight (Waters Corporation)	Brain tissue	Cocaine	Deuterated internal standard (COC-d3)	Wide-isolation window; Scan-to-scan COC-to-COC-d3 normalization	Pirman <i>et al.</i> ¹³
MALDI (60-Hz, 337-nm nitrogen laser with a spot size of 100 μm)	LIT	MALDI LTQ-XL (Thermo-Fisher Scientific)	Piglet brain	Acetyl-l-carnitine (AC)	Deuterated internal standard (acetyl-d3-carnitine) homogenously applied beneath the tissue section	MS/MS with a 6-Da wide-isolation window; Scan-to-scan AC-to-AC-d3 normalization	Pirman <i>et al.</i> ^{24, 27}
MALDI (200-Hz, 355-nm YAG laser with a spot diameter of 150 μm)	Q-IM-TOF	Synapt MALDI-Q-Ion Mobility (T-wave cell) time-of-flight (Waters Corporation)	Piglet brain	Acetyl-l-carnitine	Deuterated internal standard (acetyl-d3-carnitine)	Full-scan MS mode	Pirman <i>et al.</i> ²⁷
MALDI	TOF/TOF	Ultraflex III (Bruker Daltonics)	Peptides generated after <i>in-situ</i> tryptic digestion	Isotopically labeled standard peptide from a target protein; Homogenously applied onto the tissue after <i>in situ</i> tryptic digestion	MRM	Clemis <i>et al.</i> ²⁵	
MALDI	TOF/TOF	Ultraflex II (Bruker Daltonics)	Lung and brain tissues from rats	Drugs (imipramine and tiotropium) and endogenous compounds	Isotopically labeled standard	MS	Kallback <i>et al.</i> ²⁶

CHAPTER 5.

Quantitative MS Imaging (QMSI)

Part 2 – Quantification in MALDI: What can we learn from MALDI-SRM/MS and what can we expect for MALDI-SRM/MS imaging?



5.2.1. Introduction

Liquid chromatography coupled with mass spectrometric detection is the technique of choice for the analysis and quantification of drugs and their metabolites, or endogenous compounds, from complex biomatrices such as tissue. The general workflow in drug metabolism consists of dosing an animal and after sacrifice, organs are dissected, homogenized and analytes are extracted, prior to their injection into the LC-MS/MS system for analysis. Calibration curves are built either from tissue spiked with the substance(s) of interest or by using surrogate samples such as plasma. The final result can then finally be expressed in gram of analyte per gram of tissue. The performances expected of such LC-ESI-SRM/MS approaches are: precision and accuracy better than 15%, dynamic range of at least three orders of magnitude and labeled internal standards are required to compensate for matrix effects.

As alternative approaches to LC-SRM/MS, platforms based on matrix assisted laser desorption/ionization (MALDI)-MS/MS have been successfully developed for quantification of pharmaceuticals in biological samples. Particularly, the use of the selected reaction monitoring mode provides sufficient selectivity and sensitivity to reach performances equal to the ones achieved by LC-ESI-MS/MS. For quantification, an average of 4 spots is usually required and the calibration curves are built based on the area ratio measured for each spot between an analyte and its internal standard.³⁶ Indeed, the use of an internal standard is preferred to improve quantification.^{23-25, 37} Based on these observations, it clearly appears that using a (labeled) internal standard seems to be appropriate for performing quantification by MALDI-SRM/MS imaging and thus will have an impact on the information generated. Based on these observations, came the question: what can we expect for performing quantification by MALDI-SRM/MS imaging?

Different factors should be considered for imaging and quantification by MALDI-MS. Among them, the matrix deposition plays a major role. The spatial resolution is one of the key parameters that significantly impacts the nature of the molecular information generated from an MS imaging experiment. To illustrate briefly, Norris and Caprioli recently showed the influence of a change in spatial resolution on the quality of the resulting image, by comparison of serial mouse brain images acquired at different spatial resolutions ranging from 25 to 200 μm (Figure V- 2).³⁸ Thus, the nature of the sample and also the objectives of the study drive the design of experiments. Because increasing the spatial resolution leads to a considerable decreases of the sample throughput and sensitivity, and also increases the amount of data generated, it is mandatory that MS imaging researchers use a smart combination of spatial resolution and adequate experimental parameters.

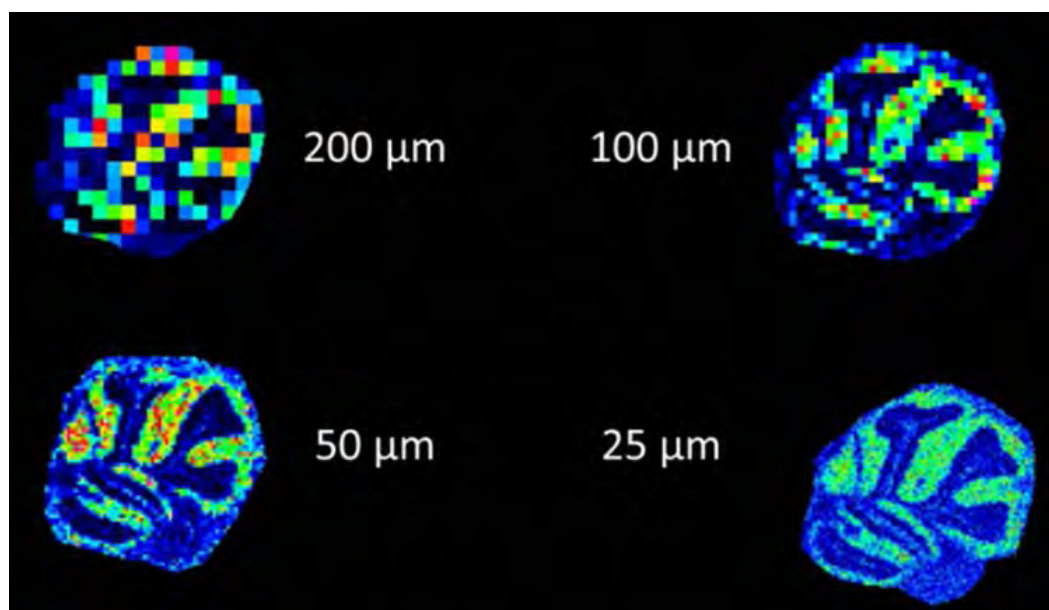


Figure V- 2. Comparison of serial mouse brain images acquired at different spatial resolutions. Ion images (m/z 6765) of mouse cerebellum imaged by MALDI IMS at spatial resolutions of 200, 100, 50, and 25 μm . From ref.³⁸

A “pixel” is the smallest measurable unit of a raster image, defined by its (x;y)-coordinates. A “voxel” is defined as a volume element, i.e. the 3D analogue of a 2D pixel. In MSI, a voxel is defined by its coordinates in the raster image and by its intensity (i.e. given by the MS signal recorded). Several instrumental parameters may affect the size of pixel. It begins with the laser beam diameter and the way of acquiring the MS images (i.e. discrete or rastering modes). When the image is acquired in the discrete mode, the pixel size is defined by the pitch set between the ablation spots (see Chapter 1, Part 2). Spot-by-spot analysis may result in several hours acquisition time.

As the throughput is one of the major issues encountered in microprobe MALDI-MS imaging approaches, a strategy consisting of continuous laser raster sampling has been introduced to improve the ion image acquisition throughput.³⁹⁻⁴⁰ The rastering mode consists in moving the sample stage laterally while maintaining a constant laser repetition rate and constant sample stage velocity. High sample stage velocity can be used and in combination with high frequency lasers (i.e. in the range of kHz), data can be generated in a very rapid fashion. This means that a rapid data collection is also required, and fast MS duty cycle acquisition modes are mandatory. This is the case for the selected reaction monitoring (SRM) mode. When using a continuous laser raster sampling, the lateral spatial resolution (x-dimension) becomes a function of the plate stage velocity and the duty cycle of the experiment, also referred as total scan time (Figure V- 3).

The lateral spatial resolution can be thus calculated as follow:

$$\text{Lateral spatial resolution} = \text{plate stage velocity} \times \text{total scan time of the experiment}$$

In SRM experiment, the total scan time (TST) is a function of the number of transition(s) monitored (n), the pause between each transition (i.e. 5 ms) and the dwell time (DT) set for each transition:

$$\text{Total scan time (s)} = n \times \text{DT(s)} + n \times 0.005$$

Vertically, the spatial resolution is defined by the distance between 2 laser line scans. The precision of the sample stage positioning also plays a role in the definition of the spatial resolution. Imaging at spatial resolution less than the size of the laser beam diameter is possible using an oversampling method.⁴¹⁻⁴²

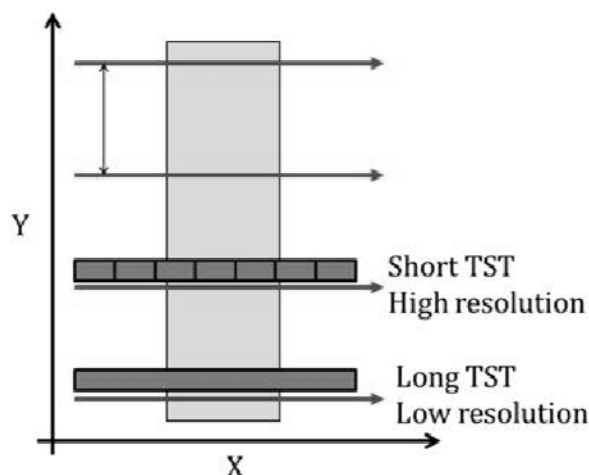


Figure V- 3. Factors influencing the resolution and the analysis time in microprobe MS imaging performed in a continuous laser raster sampling.

The objective of the following discussion is to highlight the different instrumental parameters influencing the quality of the MALDI-SRM/MS images acquired *versus* the images generated (this point will be explained later). The plate stage velocity, the duty cycle of the SRM experiments as well as the laser intensity will be considered. The importance of the data processing to generate the image and also the necessity to use a deuterated standard to normalize the images in a targeted approach for low molecular weight compounds will also be pointed out.

5.2.2. Material and methods

5.2.2.1. Chemicals and reagents

Cocaine (COC) and cocaine- d_3 (COC- d_3) were provided by Lipomed (Arlesheim, Switzerland). Methanol (MeOH, HPLC grade) was provided by Sigma-Aldrich (Buchs, Switzerland). α -cyano-4-hydroxycinnamic acid (CHCA) was provided by Fluka (Buchs, Switzerland). Formic acid (HCOOH) was purchased from Merck (Darmstadt, Switzerland). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

5.2.2.2. Preparation of standards and matrix solutions

A standard solution containing both cocaine (COC) and its deuterated analogue cocaine- d_3 (COC- d_3) was prepared at a concentration of 2 $\mu\text{g}/\mu\text{L}$ (unless otherwise stated) in ACN:H₂O:HCOOH (60:40:0.1, v:v:v) from individual stock solutions of 1 mg/mL in MeOH.

The mixture was then mixed 1:1 (v:v) with CHCA matrix solution 10 mg/mL in ACN:H₂O:HCOOH (60:40:0.1, v:v:v) and several 1 μ L of were spotted on stainless steel MALDI plate. Final amount of standards spotted onto target were of 1 pg.

5.2.2.3. MALDI Mass spectrometry imaging

Acquisitions were performed on a triple quadrupole linear ion trap mass spectrometer (AB Sciex, Concord, ON, Canada) with a MALDI source in front equipped with a frequency-tripled Nd:YAG laser 355 nm (elliptic beam shape of 100 by 200 μ m). Acquisitions were performed in positive ion ionization mode in the selected reaction monitoring mode. The SRM transitions monitored were m/z 304 > m/z 182 for cocaine (DP = 70 V ; CE = 27 eV) and m/z 307 > m/z 185 for cocaine-d₃ (DP = 70 V ; CE = 27 eV). General operating conditions were: data acquisition mode = horizontal rastering mode (following the x-dimension), repetition rate laser = 50-1'000 Hz; laser energy = 40-80 μ J; MALDI source and q0 region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q2 = 2.4x10⁻⁵ Torr (nitrogen used as collision gas); entrance potential = 10 V; quadrupole resolution was set to unit. The distance between 2 line scans, the dwell time set for each transition, the plate stage velocity and the resulting pixel sizes are detailed Table V- 2. The signals were recorded at each position of the samples and a 2D-map based on each SRM transition can be generated. In our experimental setup, the minimum step achievable in-between 2 raster lines with the motors positioning the sample stage is of 30 μ m.

The number of laser shots per pixel is calculated as follow:

$$\text{Laser shots per pixel} = \text{frequency(Hz)} \times \text{DT(s)}$$

where the frequency is defined as the number of laser shot per second, i.e. 1'000 laser shots per second at the frequency of 1'000 Hz.

Table V- 2. Description of the experiments. Two SRM transitions were monitored in each experiment. The size of the pixel defined in y corresponds to the distance between 2 line scans of the laser operating in the rastering mode. Data in bold indicate that square pixels were acquired. *Images acquired using the oversampling method.

Set of experiments	Changing parameter	Sample stage velocity	Laser		SRM method		Pixel size	
		mm/s	frequency (Hz)	shots/pixel	DT (ms)	TST (ms)	X (μm)	Y (μm)
Set A	Distance between 2 line scans	1	1'000	5	20	50	50	30*
		1	1'000	5	20	50	50	50*
		1	1'000	5	20	50	50	100
		1	1'000	5	20	50	50	200
Set B	Laser shots per pixel	1	1'000	5	5	20	20	100
		1	1'000	10	10	30	30	100
		1	1'000	20	20	50	50	100
		1	1'000	45	45	100	100	100
		1	1'000	95	95	200	200	100
Set C	Acquisition of square pixels	1	1'000	20	20	50	50	50*
		2	1'000	20	20	50	100	100
		3	1'000	20	20	50	150	150
		4	1'000	20	20	50	200	200
Set D	Plate speed	1	1'000	20	20	50	50	100
		2	1'000	20	20	50	100	100
		3	1'000	20	20	50	150	100
		4	1'000	20	20	50	200	100
Set E	Frequency (with same MS method)	1	1'000	45	45	100	100	100
		1	500	22.5	45	100	100	100
		1	200	9	45	100	100	100
		1	100	4.5	45	100	100	100
		1	50	2.25	45	100	100	100
Set F	Frequency (with number of shots per pixels)	1	1'000	5	5	20	20	100
		1	500	5	10	30	30	100
		1	200	5	25	60	60	100
		1	100	5	50	110	110	100
		1	50	5	100	210	210	100

5.2.2.4. Software for data acquisition and processing

The MALDI source and its laser were controlled using a customized M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and for data collection. PeakView software (v.1.0.0.3, AB Sciex) was used for raw data processing (.wiff files). A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files (.wiff format) into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex), used for SRM/MS images generation and

processing. Tissue View software offers several mathematical functions available for processing MS imaging. To generate the so-called “normalized images” for cocaine, the function “divide” in the “tools” menu was used. In the case of MS image (.img file) acquired in the selected reaction monitoring mode, an .img file is created for each SRM transition. The “divide” function consists in a normalization of one image (i.e. based on the SRM trace for cocaine) by another one “pixel-by-pixel”. The region(s) of interest is(are) then drawn in Tissue View on this new image processed by the software and the data extracted and imported in Excel for further statistical calculations.

5.2.3. Results and discussion

The MSI platform used in this work is a triple quadrupole linear ion trap (QqQ_{LIT}) mass analyzer fitted with a MALDI source operated at intermediate pressure (i.e. 1 Torr). The MALDI source is equipped with a Nd:YAG solid state laser capable of pulse repetition rates up to 1 kHz and continuous laser raster sampling for high-throughput data generation. The laser beam has an elliptic shape of 100 x 200 μm , in the x- and y-dimensions, respectively. The mass analyzer is operated in the selected reaction monitoring (SRM) mode for fast data collection. To generate image with pixel size smaller than the beam of the laser, the oversampling method is used.^{40, 43-44}

5.2.3.1. Effect of oversampling on the signal intensity

To characterize the possible effect on the signal measured due to oversampling, all the parameters have been set to fixed values, with the obvious exception of the distance between 2 laser line scans, i.e. 30 μm , 50 μm , 100 μm and 200 μm (i.e. Set of experiments A, from (Table V- 2). As the laser beam has a shape of 100 x 200 μm in horizontal rastering mode, the experiments using a 30 μm and 50 μm -distances between two raster lines have been performed with the oversampling method. No oversampling was used when using incremental steps of the sample stage of 100 μm and 200 μm . The resulting MS images are displayed Figure V- 4.

a) Display modes available in Tissue View software

In the image settings, two display modes are available in Tissue View software: the “interpolate” and the “voxel” mode. Figure V- 4 shows an example of the same MALDI spots displayed in the two modes (i.e. Figure V- 4.a and Figure V- 4.b for the “interpolate” mode and the “voxel” mode, respectively). It is clearly visible on the images that the “interpolate” mode slightly smoothes the image generated. This results than small features can be neglected. This reflects the importance of the way to display the image, in function of the target, and the influence on the information given. To better represent the “true” distribution of the analytes within the spot, the next images of this study will be generated using the “voxel” mode, where each pixel is displayed with its own intensity (or its value after pixel-by-pixel normalization the images with the deuterated analog).

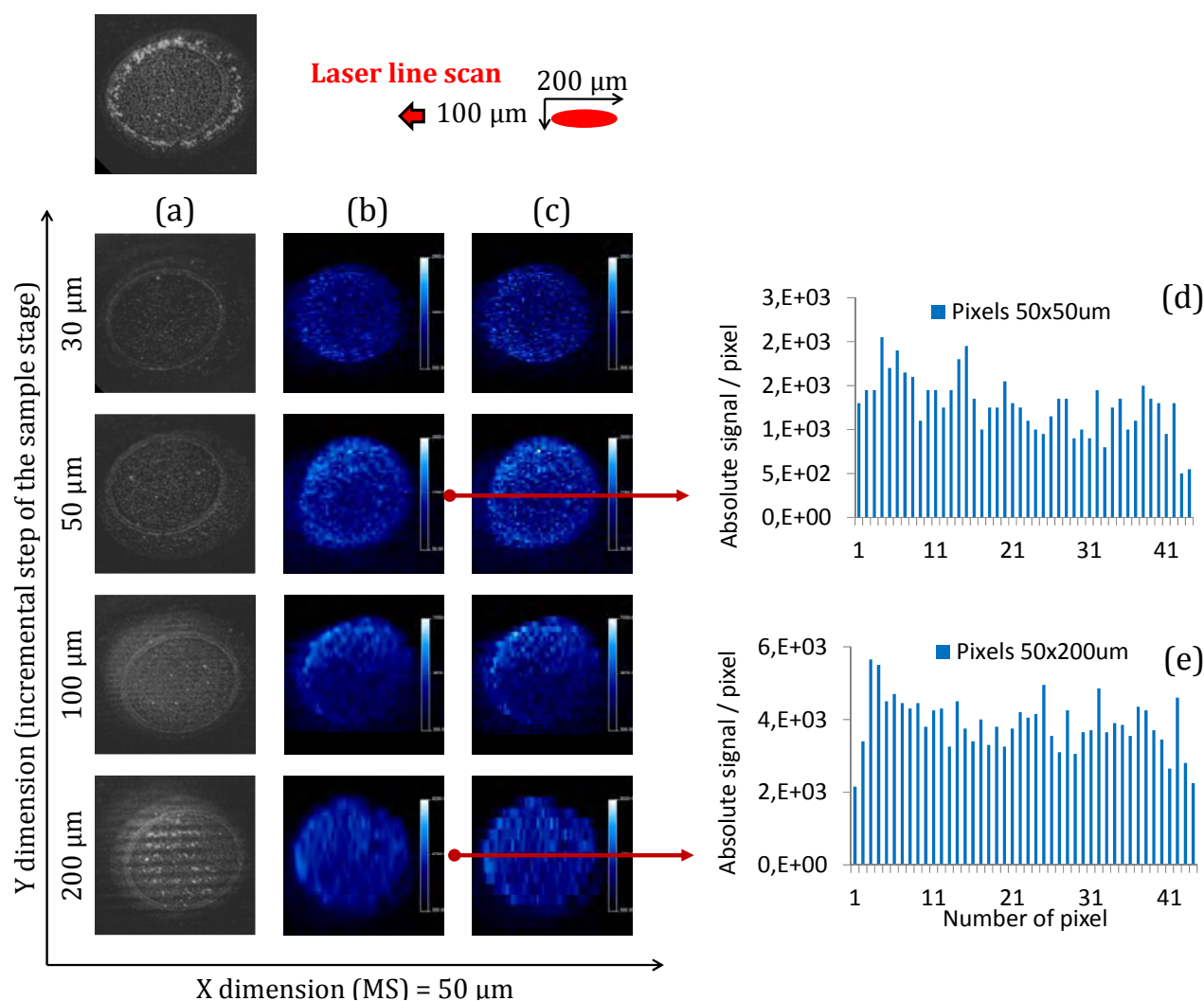


Figure V- 4. (a) Photographs of MALDI spots before (1st upper spot of the column) and after the laser passed through the spots, performed using 30 μm, 50 μm, 100 μm and 200 μm incremental steps of the sample stage. MALDI-SRM/MS images based on the SRM trace of cocaine generated with Tissue View software and displayed using the (b) “interpolate” and (c) “voxel” mode. Pixel to pixel variability in one raster line from images acquired and generated with pixel size of (d) 50 x 50 μm (oversampling) and (e) 50 x 200 μm. Data are given for cocaine (absolute area) for 1 pg of standard spotted (from Set A). Each MALDI-SRM-based image is given with its own scale intensity. The maximum intensities were (arbitrary units given by Tissue View software): 2900 for the pixels of 50 x 30 μm; 3500 for the pixels of 50 x 50 μm; 7050 for the pixels of 50 x 100 μm and 9020 for the pixels of 50 x 200 μm. The minimum intensity was set 300 for all images.

b) Intra- and inter-spot variability measured with and without the oversampling method

A MALDI spot is defined by its population, i.e. the number of pixels in a defined area, which will obviously increase when the size of the pixel decreases, for the same surface. To obtain the following results, regions of interest (ROI) were defined as follow: a circle was manually drawn to cover the surface of a single MALDI spot. The same surface was used for the 4 spots acquired under the same conditions.

The first observation is the pixel to pixel variability in single line scan acquired along a MALDI spot. This variability can be up to 26% as shown for the image acquired and

generated with pixel sizes of 50 x 50 μm or 19% for the image with pixel sizes of 50 x 200 μm (Figure V- 4.d and e, respectively). This variation can be improved down to 20% by the normalization with the internal standard cocaine- d_3 in both experimental conditions (data not shown).

Table V- 4 and Figure V- 5 summarize the intra and inter-spot variability measured between four MALDI spots (ROI drawn in TissueView software) and compare four experimental conditions where the only variable is the distance between two raster lines (i.e. Set A, Table V- 2). Decreasing the step used to move the sample stage in the vertical direction (i.e. Y-axis) leads to a decrease of the absolute signal for both cocaine and cocaine- d_3 . This can be explained because of the desorption of a surface that has been previously partially rastered by the laser beam. Results traduce a high intra-spot variability (i.e. within the range 20-40%, regarding the absolute signal give for COC and COC- d_3 , without normalization) whatever the condition (Table V- 3). The use of internal standard does not allow compensating for the intra-spot variability in all cases, and even increases the variability. The inter-spot variability varies from 8-16% and 5-6% for COC and COC- d_3 , respectively (absolute area). The use of internal standard to normalize the signal pixel-by-pixel allows for maintaining this variability below 15% (Table V- 4). It results from these first experiments that the oversampling method does not affect the variability of the signal measured, but lowers the absolute signal. Also, regarding the lower inter-spot variability (compared to the pixel-to-pixel variability measured within a single spot), it results that there is a minimum number of pixels to use for performing quantification. This latest point will be discussed later in § 5.3.2.6.

Table V- 3. Intra-spot variability measured within MALDI spots when moving the sample stage with incremental steps of 30, 50, 100 and 200 μm (corresponds to the Set of experiment A). The results give the variation of the signal given by each pixel within one population (= variation in one spot). 4 MALDI spots have been measured per condition. *The variation of the signals given for COC and COC- d_3 is based on the absolute signal of each pixel in the corresponding image. **The ratio COC/COC- d_3 is given after pixel-by-pixel normalization calculated via Tissue View software.

Pixel size in Y	Area	Population	Variation within a population (= intra-spot variation, RSD%)		
			Signal for COC*	Signal for COC- d_3 *	Ratio**
30 μm	170 mm^2	1859	24 to 27% (25%, n=4)	34 to 37% (35%, n=4)	50 to 73% (62%, n=4)
50 μm	179 mm^2	1182	25 to 26% (26%, n=4)	31 to 40% (36%, n=4)	43 to 130% (91%, n=4)
100 μm	179 mm^2	625	28 to 31% (30%, n=4)	32 to 34% (33%, n=4)	28 to 38% (32%, n=4)
200 μm	179 mm^2	339	20 to 23% (22%, n=4)	24 to 34% (29%, n=4)	38 to 107% (70%, n=4)

Table V- 4. Inter-spot variability measured for 4 MALDI spots when moving the sample stage with incremental steps of 30, 50, 100 and 200 μm (Set A). One spot correspond to one ROI drawn in TissueView software. *The variation of the signals given for COC and COC-d₃ is based on the absolute signal of each pixel in the corresponding image. **The ratio COC/COC-d₃ is given after pixel-by-pixel normalization.

Pixel size in Y	Area	Population	Inter-spot variation (n=4), RSD%, n=4)		
			Signal for COC*	Signal for COC-d ₃ *	Area ratio**
30 μm	170 mm ²	1859	14%	5%	9%
50 μm	179 mm ²	1182	8%	6%	14%
100 μm	179 mm ²	625	9%	5%	9%
200 μm	179 mm ²	339	16%	5%	4%

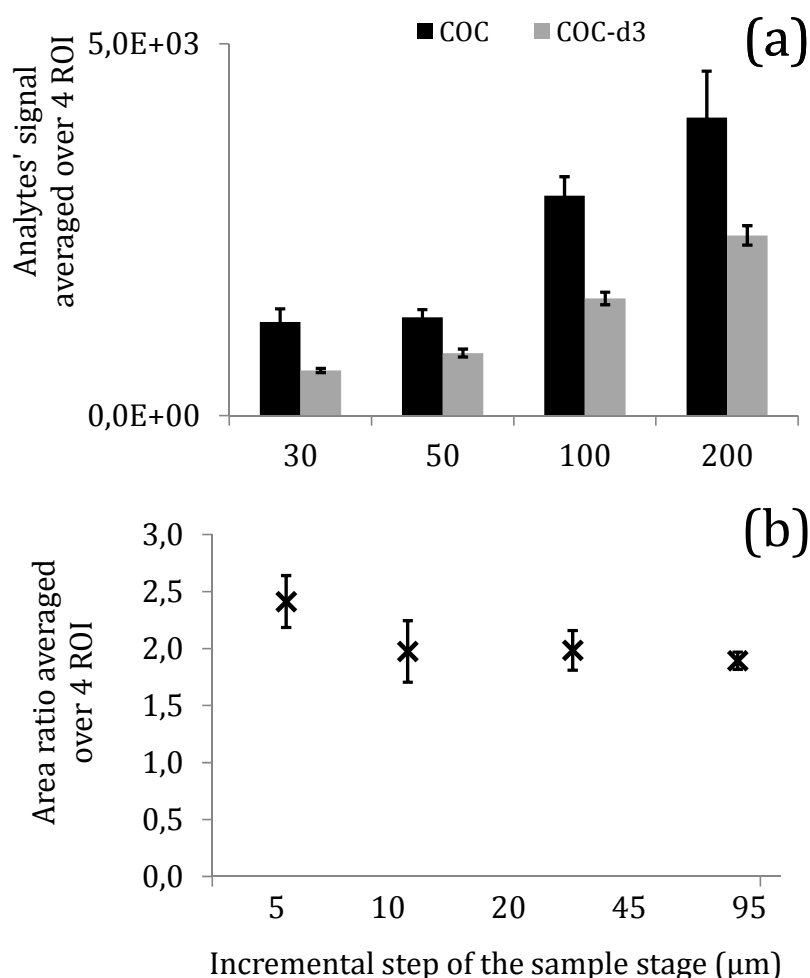


Figure V- 5. Diagrams showing the effect of moving the sample stage in the vertical (Y-axis) direction by steps of 30 μm , 50 μm , 100 μm and 200 μm on the signal intensity for (a) cocaine (COC) and cocaine-d₃ (COC-d₃) (the data correspond to the Set of experiment A). (b) The ratio COC/COC-d₃ is given after normalization of the signal from cocaine by the signal of cocaine-d₃. Data generated after drawing ROI of the size of MALDI spot with TissueView. Error bars represent ± 1 standard deviation (n=4 MALDI spots per condition). Conditions of acquisition: pixel size in the x-dimension = 50 μm ; DT = 50 ms; sample stage velocity: 1 mm/s. The number of laser shot per pixel is 5.

5.2.3.2. Influence of the laser energy on image generated

As it was previously demonstrated that the laser intensity and the spot size have influence on the desorption/ablation of molecules and ions in MALDI experiments,⁴⁵ This effect will have undeniably affect MS imaging experiments results. Figure V- 6 displays images generated after the ionization of the surface of a MALDI spot containing cocaine and its deuterated analog with a 1 kHz-laser at different energies ranging from 40 to 80 μJ . The experiments were performed under the following conditions: pixel size = 100 μm x 100 μm ; DT = 20 ms; sample stage velocity: 2 mm/s. The number of laser shots per pixel is 20.

Increasing the laser energy increases the absolute signal for cocaine (Figure V- 6.f). This correlates previous assumption that high laser power can be used on a MALDI on a triple quadrupole platform that decouples the desorption/ionization from the mass analysis.⁴⁶ The variation measured varies from 6% (at 60 μJ) to 15% (at 70 μJ). The normalization of the signal by COC-d₃ allows to decrease the variation down to 2-3% (at 40-50-60 μJ), while the variation is of 10% and 14% at 70 μJ and at 60 μJ , respectively.

However, increasing the laser energy has a dramatic effect on the quality of the MS image generated as it leads to an increase of the “snowplow effect”. The “snowplow effect” is viewed as a spread of the matrix crystals and thus delocalizes the analytes from their original position within the spot. This effect is caused by the laser path at too high energy and is more significant above laser energy of 60 μJ (Figure V- 6.a-e). Although it also lead to an increase of the absolute signal measured for cocaine (Figure V- 6.f), this must be avoided in any imaging experiments, as it completely hamper the quality of the image generate and loose the spatial distribution of the sample.

5.2.3.3. Influence of the number of shots per pixels on the signal intensity

To understand the relationship between the number of shots per pixel on the signal intensity, all the parameters have been set to fixed values, except the dwell time set for each experiment set so that 5 images could be generated with 5 to 95 laser shots per pixel (i.e. Set of experiments B, from Table V- 2). This will have a direct effect on the lateral resolution of the image generated. The distance between two raster lines is set to 100 μm (no oversampling).

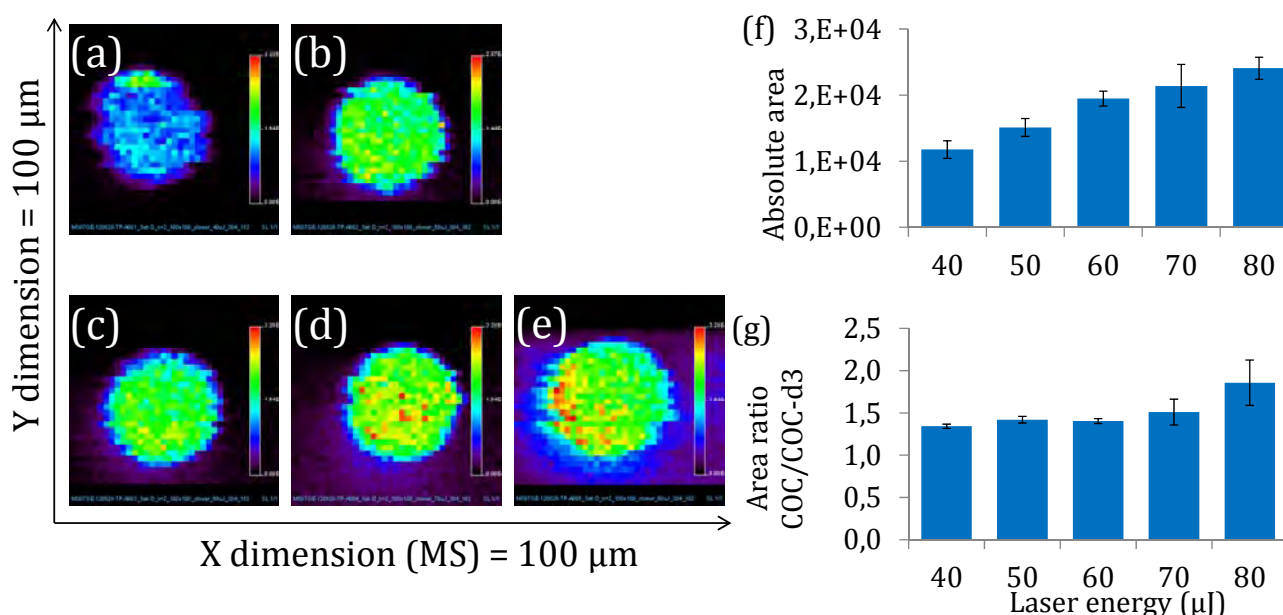


Figure V- 6. Influence of the laser energy on MS images generated. The laser energy applied is: (a) 40 μJ; (b) 50 μJ; (c) 60 μJ; (d) 70 μJ and (e) 80 μJ. (f,g) Diagrams showing the effect of the laser energy on the (f) absolute signal measured for cocaine and (g) area ratio after normalization with the signal given by COC-d3. Error bars represent ± 1 standard deviation calculated over the mean of 4 MALDI spots for each condition. The signals were extracted from regions of interest (ROI) drawn in Tissue View software so that 1 ROI = 1 MALDI spot. Conditions of acquisition: pixel size = 100 μm x 100 μm; DT = 20 ms; sample stage velocity: 2 mm/s. The number of laser shots per pixel is 20. MALDI-SRM/MS image based on the SRM trace for cocaine (COC; m/z 304 > m/z 182) and displayed in the voxel mode in Tissue View software. Data for cocaine, absolute area, 10 pg of standard were spotted.

Table V- 5. Intra-spot variability measured within MALDI spots for 5 different conditions where the number of laser shots per pixel varies from 5 to 95 (Set of experiment B). The results give the variation of the signal given by each pixel within one population (= variation in one spot). 4 MALDI spots were measured per condition. *The variation of the signals given for COC and COC-d₃ is based on the absolute signal of each pixel in the corresponding image. **The ratio COC/COC-d₃ is given after pixel-by-pixel normalization.

Shot per pixels	Area (mm ²)	Population	Variation within a population (= intra-spot variation, RSD%)		
			Signal for COC*	Signal for COC-d ₃ *	Ratio**
5	176	1420	51 to 54% (53%, n=4)	82 to 86% (83%, n=4)	99 to 103% (100%, n=4)
10	176	956	29 to 37% (32%, n=4)	59 to 59% (26%, n=4)	81 to 86% (83%, n=4)
20	176	566	27 to 35% (30%, n=4)	38 to 43% (39%, n=4)	77 to 116% (85%, n=4)
45	176	255	20 to 28% (24%, n=4)	23 to 26% (25%, n=4)	36 to 41% (38%, n=4)
95	176	134	16 to 29% (22%, n=4)	18 to 22% (21%, n=4)	21 to 37% (31%, n=4)

Table V- 6. Inter-spot variability measured for 4 MALDI spots for 5 different conditions where the number of laser shots per pixel varies from 5 to 95 (Set B). *The variation of the signals given for COC and COC-d₃ is based on the absolute signal of each pixel in the corresponding image. **The ratio COC/COC-d₃ is given after pixel-by-pixel normalization.

Shots per pixel	Population	Inter-spot variation (n=4), RSD%, n=4)		
		Signal for COC*	Signal for COC-d ₃ *	Ratio**
5	1684	11%	5%	11%
10	1187	9%	3%	7%
20	666	18%	2%	21%
45	351	8%	7%	7%
95	190	11%	3%	8%

The intra-spot variability varies from 16 to 54% for cocaine. Increasing the number of laser shots per pixels above 45 lowers the variability below 30% for both analytes. Here again, the normalization by COC-d₃ does not allow correcting for the intra-spot variability. Interestingly, the processing of ROI (i.e. so that 1 ROI = 1 MALDI spot) and the average of 4 spots allow to improve the reproducibility and inter-spot variability is below 10% in the best cases, and even below 5% after normalization by COC-d₃. for all conditions. This emphasizes the fact that quantification cannot be performed on a single pixel, but should consider a minimum number of pixels, and also a number of laser shots per pixels higher than 45 in the current instrumental setup.

Figure V- 7a. shows a decrease of the signal intensity for both COC and COC-d₃ with the increase of the number of laser shot per pixels. When smaller pixels are acquired, the risk of generating two adjacent pixels with very different signals (e.g. one pixel "on" and one pixel "off") is greater than when larger pixels are acquired. The acquisition of larger pixel will smooth this effect, and by integrating smaller pixels with different signals (i.e. 95 laser shots = 19X 5 laser shots), this will lead to a decrease of the absolute signal per pixel measured with 95 laser shots *versus* 5 laser shots. On the other hand, increasing the population per ROI (i.e. per spot) with less laser shots per pixel improves the precision of the measures, which explains why the lowest variation is obtained when smaller pixels are acquired.

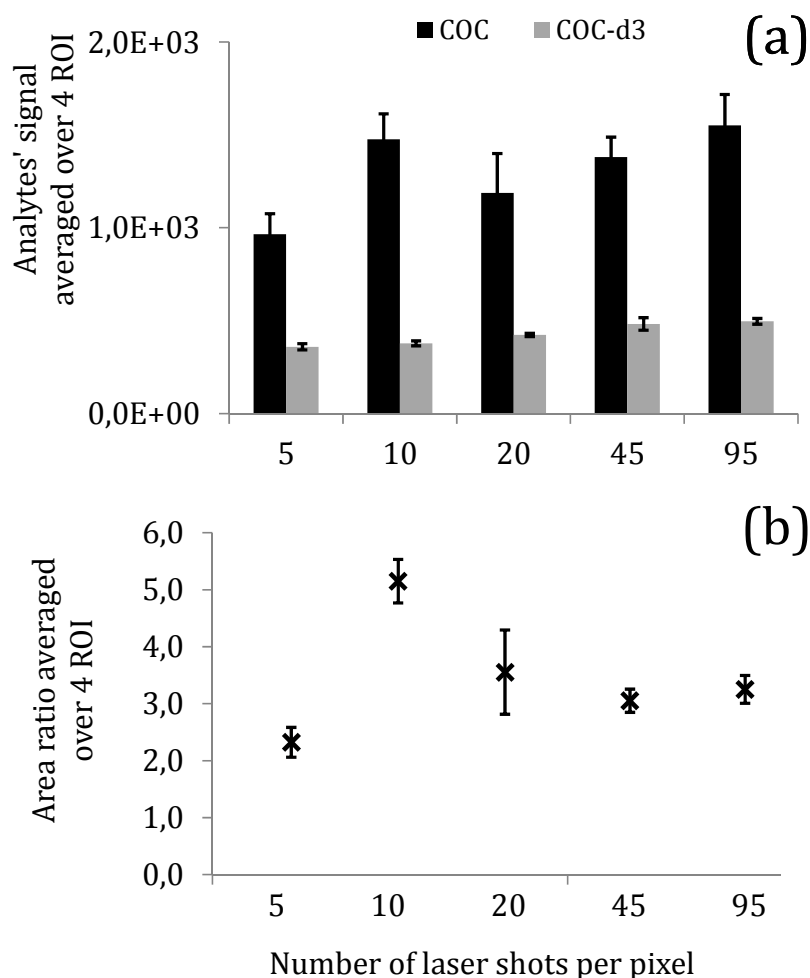


Figure V- 7. Diagrams showing the effect of laser shots per pixel on the signal intensity for (a) cocaine and cocaine-d₃ (Set of experiment B). (b) The ratio COC/COC-d₃ is given after normalization of the signal from cocaine by the signal of cocaine-d₃. Data are generated after drawing ROI of the size of MALDI spot. Error bars represent ± 1 standard deviation (n=4 MALDI spots per condition). Conditions of acquisition: pixel size in the y-dimension = 100 μ m; DT = 5 to 95 ms; sample stage velocity: 1 mm/s.

5.2.3.4. Relationship between the signal intensity and the sample stage velocity

To understand the relationship between the signal intensity and the sample stage velocity, the repetition rate laser, the laser power, the number of laser shots/pixel and the step size in-between two raster lines (Y-step) must be constant. The fixed parameters are: frequency = 1 kHz; laser power = 50 μ J; number of shots/pixel = 20; Y-step = 100 μ m. The sample stage was moved at different velocities ranging from 1 mm/s to 4 mm/s (Set of experiments D, Table V- 2). Table V- S1 displays the inter-spots variabilities calculated for the measurements of 4 MALD spots per velocity conditions. The overall variability is better than 20%, but can be below 5% in the best conditions (i.e. sample stage velocity of 4 mm/s and signal of cocaine normalized by COC-d₃). Results display a loss of signal at slower sampling speed for cocaine and correlate previously published data by Spraggins and Caprioli.⁴⁰ Improving signal intensity at higher sample stage velocity means that severe oversampling may be a problem at slower rates. Increasing the repetition rate to acquire images quickly could be envisaged to reduce substantial oversampling.⁴⁰ However, while the signal

recorded for COC-d₃ is equivalent to the signal for COC (this is expected when the two analytes are mixed at the same concentration). There is no explanation so far, except that such condition promotes the ionization of COC at the detriment of the ionization of COC-d₃. But at the same laser energy for the same conditions, it is unlikely that this is the right reason.

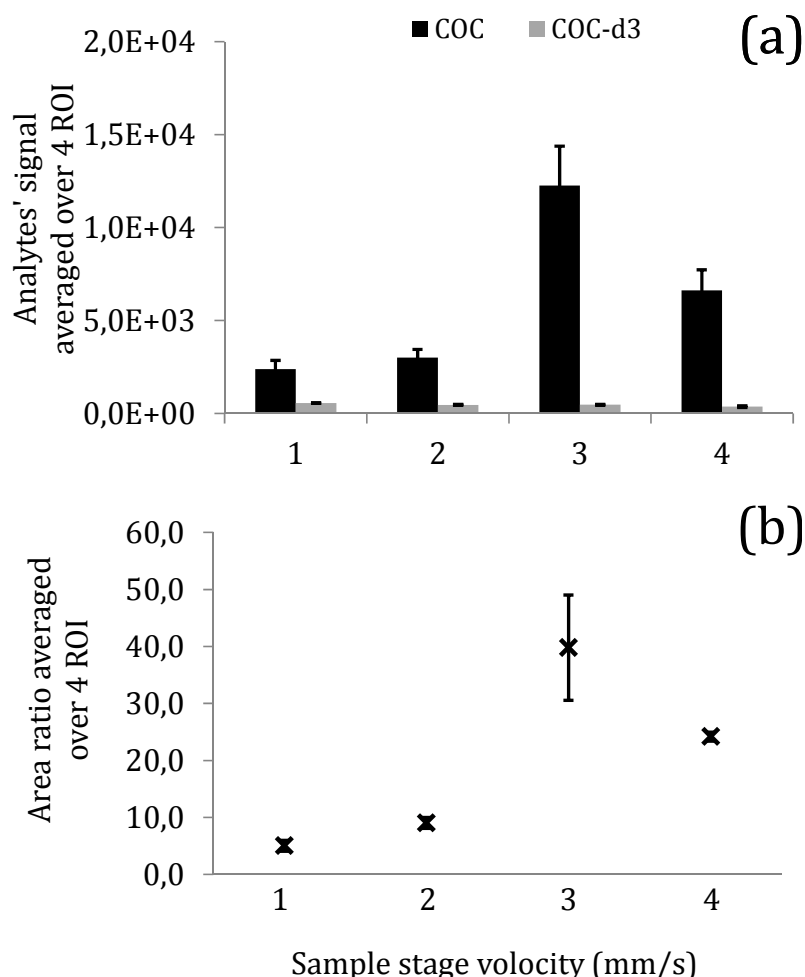


Figure V- 8. Cocaine and cocaine-d₃ signal intensity as a function of the sample stage velocity in a continuous raster sampling for (a) cocaine and cocaine-d₃ (Set of experiment D). (b) The ratio COC/COC-d₃ is given after normalization of the signal from cocaine by the signal of cocaine-d₃. Data are generated after drawing ROI of the size of MALDI spot. Error bars represent ± 1 standard deviation (n=4 MALDI spots per condition).

5.2.3.5. Influence of the laser frequency

This part focuses on the effect of the laser frequency on the signal acquired and the resulting MALDI-SRM/MS images. For that purpose, two set of experiments were carried out, namely Set E and F (see Table V- 2), where the laser frequency varied from 50 to 1'000 Hz. For each experiment, the laser energy was kept constant at 50 μ J and calibrated prior the analysis using a power/energy meter (EPM1000, Coherent, Portland, OR) equipped with a pyroelectric energy sensor (J25LP-3A, Coherent, Portland, OR).

The experiments in Set E are performed at constant sample stage velocity (i.e. 1 mm/s), the same SRM method was used for all conditions (i.e. DT = 45 ms), leading to a variation of the number of laser shots per pixel in function of the laser frequency used, i.e. 2.25, 4.5, 9, 22.5,

45 and 95 laser shots per pixel at 50, 100, 200, 500 and 1'000 Hz, respectively. The experiments in Set F are performed at constant sample stage velocity and varying laser frequency, the SRM method was adjusted in order to maintain the number of laser shots per pixel to 5.

Increasing the laser energy at the same time than the number of laser shots per pixels leads to an increase of the overall sensitivity for both COC and COC-d₃ (Figure V- 10A). The same observations can be made when a constant number of 5 laser shots per pixels is maintained for all experiments (Figure V- 10B). This means that in that specific case, the laser frequency has a greater influence on the signal measured than the number of laser shots set per pixel. Also, increasing these two parameters has a significant influence on the pixel-to-pixel variability (i.e. intra-spot variability). This variability has been improved from 71% at 50 Hz down to 18% at 1'000 Hz for cocaine (absolute signal); from 292% at 50 Hz down to 29% at 1'000 Hz for COC-d₃ (absolute signal) and also from 345% down to 39% considering the COC/COC-d₃ ratio (Set E, Table V- S2). The same trendline is observed when increasing the laser frequency up to 1'000 Hz while the number of laser shots per pixels is kept constant (i.e. 5) for all the laser energy employed; however, in this specific case, the overall intra-spot variation is above 40% (Set F, Table V- S4), which is not satisfying for performing quantitation.

The overall inter-spot variability is better than 20%, and is expected to be better than 15% when operating the laser at a frequency of 1'000 Hz and a number of 95 laser shots per pixels (Set E, Table V- S3) and better than 10% at a laser frequency of 1'000 Hz and a constant number of 5 laser shots per pixels (Set F, Table V- S5). In conclusion, higher laser frequency helps to minimize the variability and is required for performing quantification.

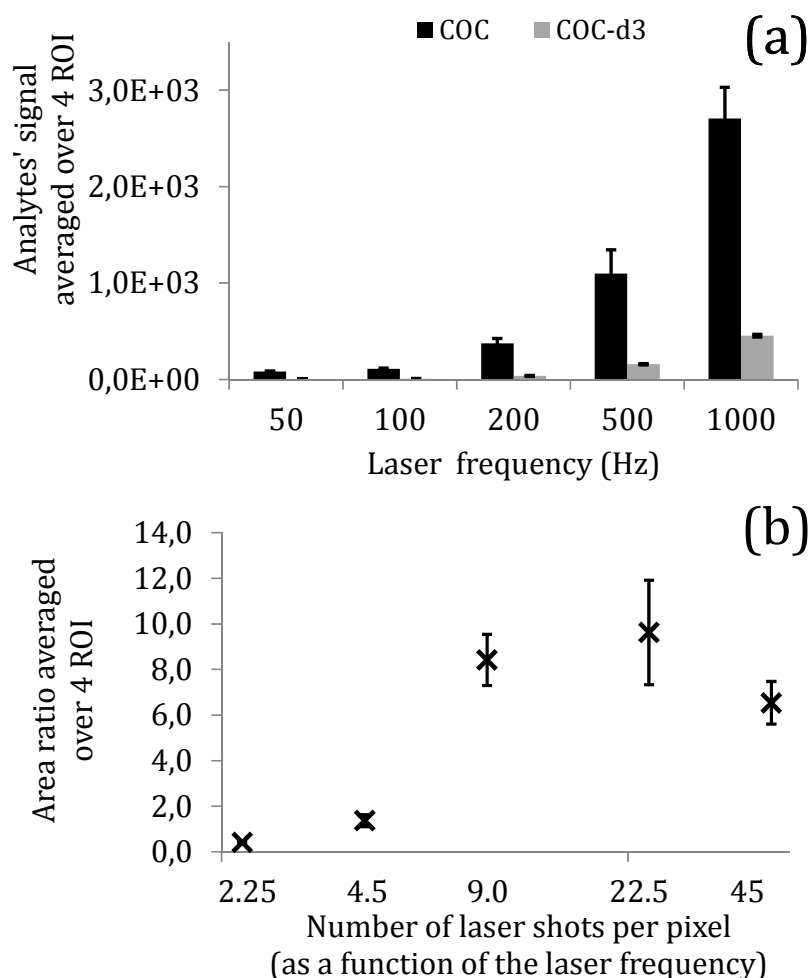


Figure V- 10A. Cocaine and cocaine-d₃ signal intensity as a function of the laser frequency for (a) cocaine and cocaine-d₃ (Set of experiment E). (b) The ratio COC/COC-d₃ is given after normalization of the signal from cocaine by the signal of cocaine-d₃. Data are generated after drawing ROI of the size of MALDI spot. Error bars represent ± 1 standard deviation (n=4 MALDI spots per condition). The same SRM method was used for all conditions, leading to a variation of the number of laser shots per pixel, which are indicated in (b), as a function of the laser frequency.

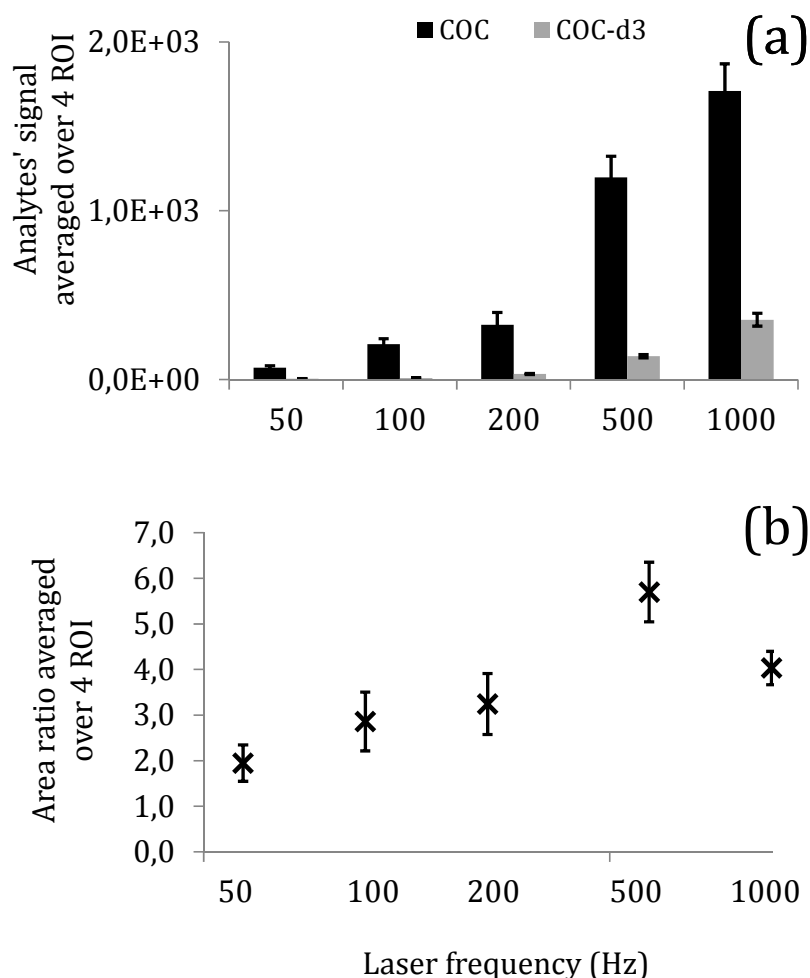


Figure V- 10B. Cocaine and cocaine-d₃ signal intensity as a function of the laser frequency for (a) cocaine and cocaine-d₃ (Set of experiment F). (b) The ratio COC/COC-d₃ is given after normalization of the signal from cocaine by the signal of cocaine-d₃. Data are generated after drawing ROI of the size of MALDI spot. Error bars represent ± 1 standard deviation (n=4 MALDI spots per condition). In order to get the same number

5.2.3.6. Importance and effect of the data processing on the generated image

The importance of the display mode used to generate an image was already pointed in § 5.2.3.1.a. The objective of this part is to assess the data quality of high resolution image (i.e. pixels of 50 x 50 μm acquired) *versus* low resolution image (i.e. pixels of 200 x 200 μm acquired) but acquired in a faster fashion. For that purpose, the data processing was made on samples from the Set of experiments C (Table V- 2). It is noteworthy that this set of data consists in 2 variables, namely the sample stage velocity and the distance between two raster lines, which have been set in order to generate square pixels, i.e. 50 x 50 μm ; 100 x 100 μm ; 150 x 150 μm and 200 x 200 μm . This means that this study cannot explain which one of the two variables affect the final results. The number laser shots per pixels remains however constant from one analysis to another. Two types of images will be compared: low resolution images (i.e. pixels of 100 x 100 μm and 200 x 200 μm) acquired in a relatively rapid fashion *versus* low resolution images but processed from high resolution images. As a result, 4 images are considered (see Figure V- 9):

- (a) The image with pixels of 100 μm x 100 μm acquired as follow: sample stage velocity: 2 mm/s; distance between two raster lines: 100 μm ;
- (b) The image displayed with pixels size of 100 x 100 μm , where each pixel corresponds to an average of 4 pixels from the image with pixels of 50 μm x 50 μm acquired as follow: sample stage velocity: 1 mm/s; distance between two raster lines: 50 μm ;
- (c) The image with pixels of 200 μm x 200 μm acquired as follow: sample stage velocity: 4 mm/s; distance between two raster lines: 200 μm ;
- (d) The image displayed with pixels size of 200 x 200 μm , where each pixel corresponds to an average of 16 pixels from the image with pixels of 50 μm x 50 μm acquired as follow: sample stage velocity: 1 mm/s; distance between two raster lines: 50 μm ;

The resulting images are shown Figure V- 10 (based on the absolute signal for cocaine) and Figure V- 11B (based on the absolute signal for cocaine normalized by the signal of COC-d₃).

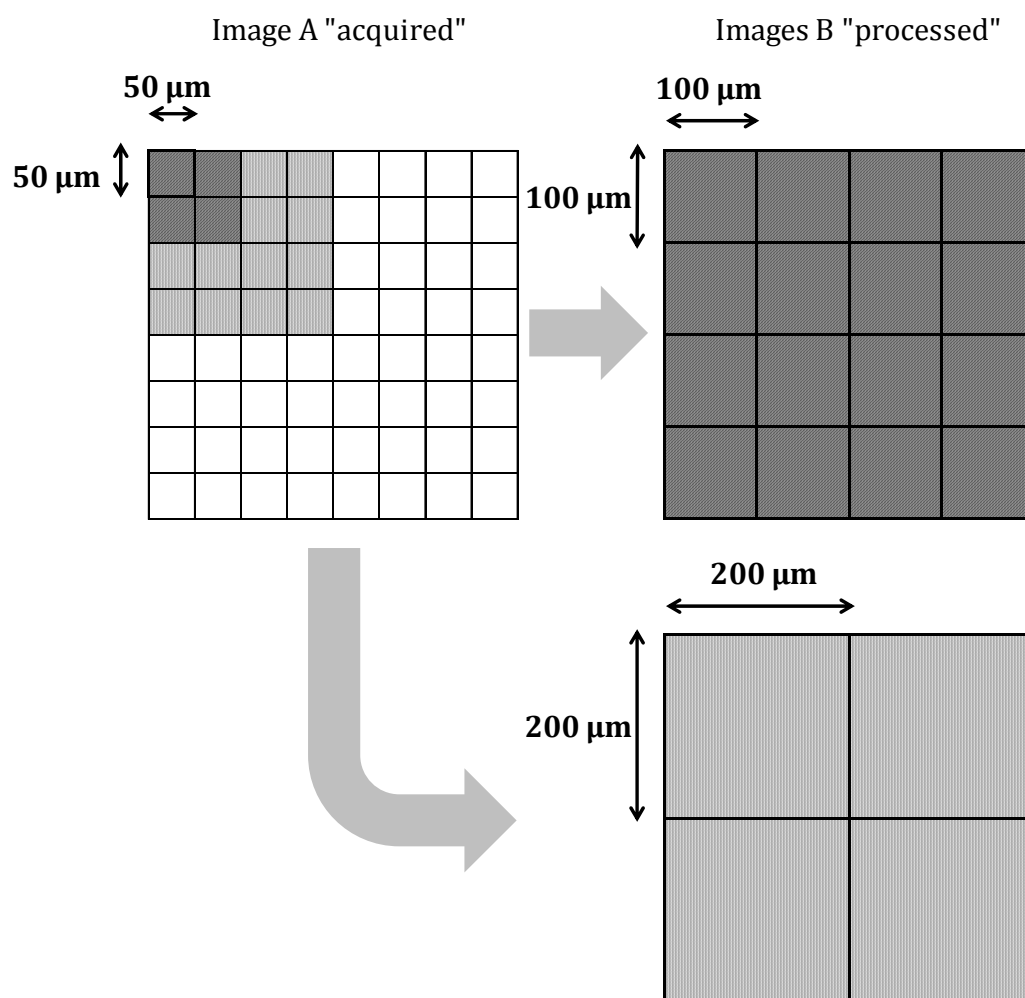


Figure V- 9. Schemes showing the generation of 2 images with pixels size of 100 x 100 μm and 200 x 200 μm (Images B) after processing of the same Image A acquired with smaller pixels (i.e. size of 50 x 50 μm). The experimental conditions correspond to the Set of experiments C to acquire square pixels.

The first observation of the images displayed Figure V- 10 and Figure V- 11B is a more homogeneous response of the signals (both absolute and normalized signals for cocaine) after processing of image acquired at high resolution, but with 2X or 4 X lower sample stage

velocities. The processing smoothes the signal over 4 or 16 pixels (to generate the pixels of 100 x 100 μm and 200 x 200 μm , respectively). It results a better reproducibility (with relative standard variation) is achieved at low acquisition velocity and variation after processing is better than 10%. Note that square regions of interest were drawn to generate Figure V- 12 and Figure V- 13. Averaging the signals over a large number of pixels may include pixels at the limits of the spot, where the signal is less intense compared to the center of the spot. This will lead to an increase of the variability of the signal measured with the increase of the number of pixels averaged. Drawing regions of interest to average signals over 5-10 pixels instead of comparing the signals pixel-by-pixel appears more suitable for performing quantitative analysis with less than 10% of signal variability. This converges with previous approaches making use of standard solution spotted onto/beneath tissue sections, and processed after drawing ROI to further calculate the amount of drug per weight of tissue (see Chapter 5, Part 1).^{15, 17-18, 30}

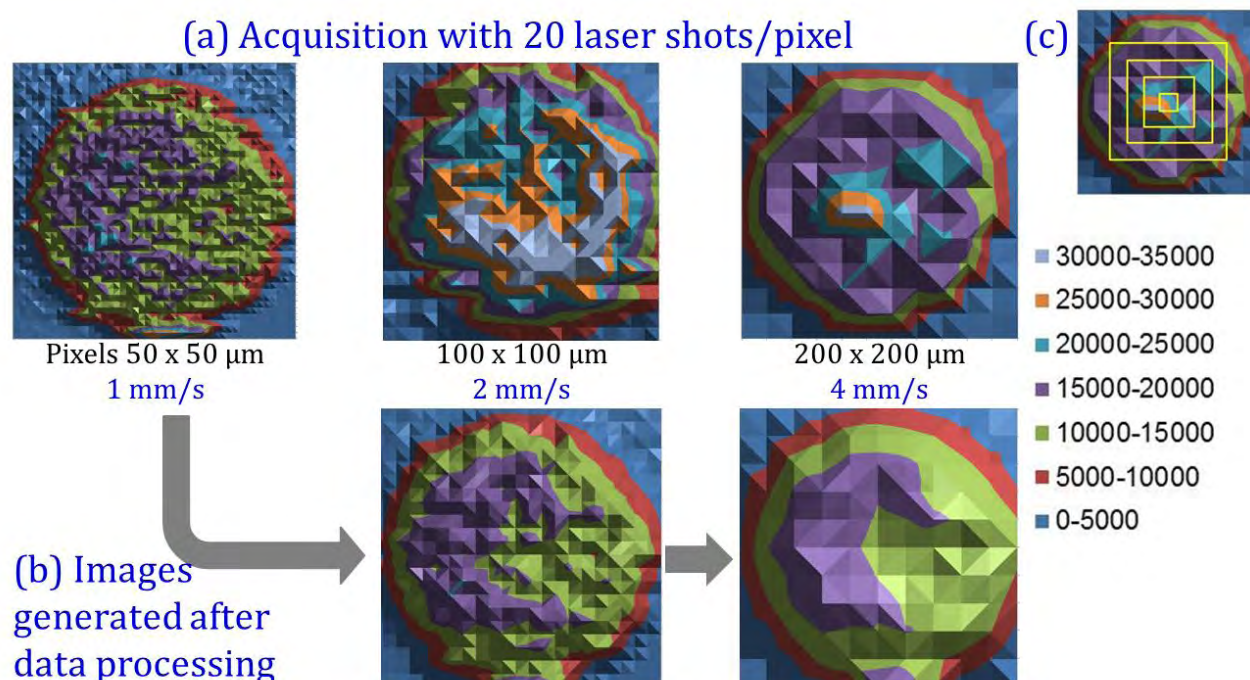


Figure V- 10A. (a) Images generated after MALDI-SRM/MSI with experimental parameters set to acquire images with pixel sizes of 50 x 50 μm , 100 x 100 μm and 200 x 200 μm . (b) Images with pixels of 100 x 100 μm and 200 x 200 μm generated after processing of the image with pixels of 50 x 50 μm . Images are based on the absolute signal measured for cocaine. (c) Schematic representation of the square area used to generate the graphs Figure V- 12 and Figure V- 13.

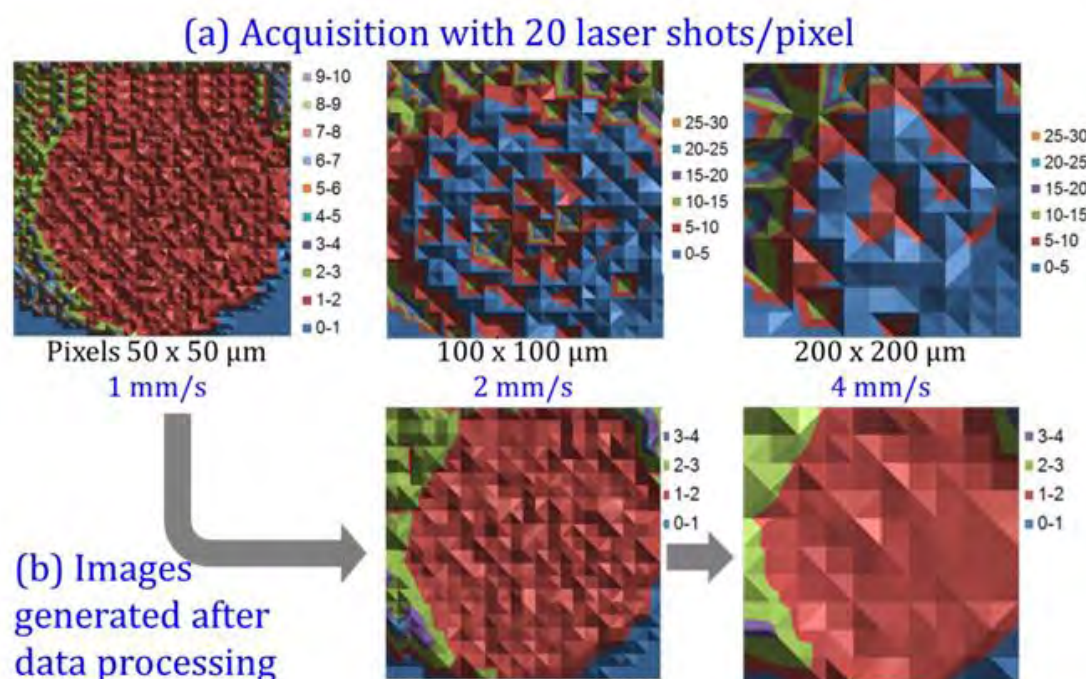
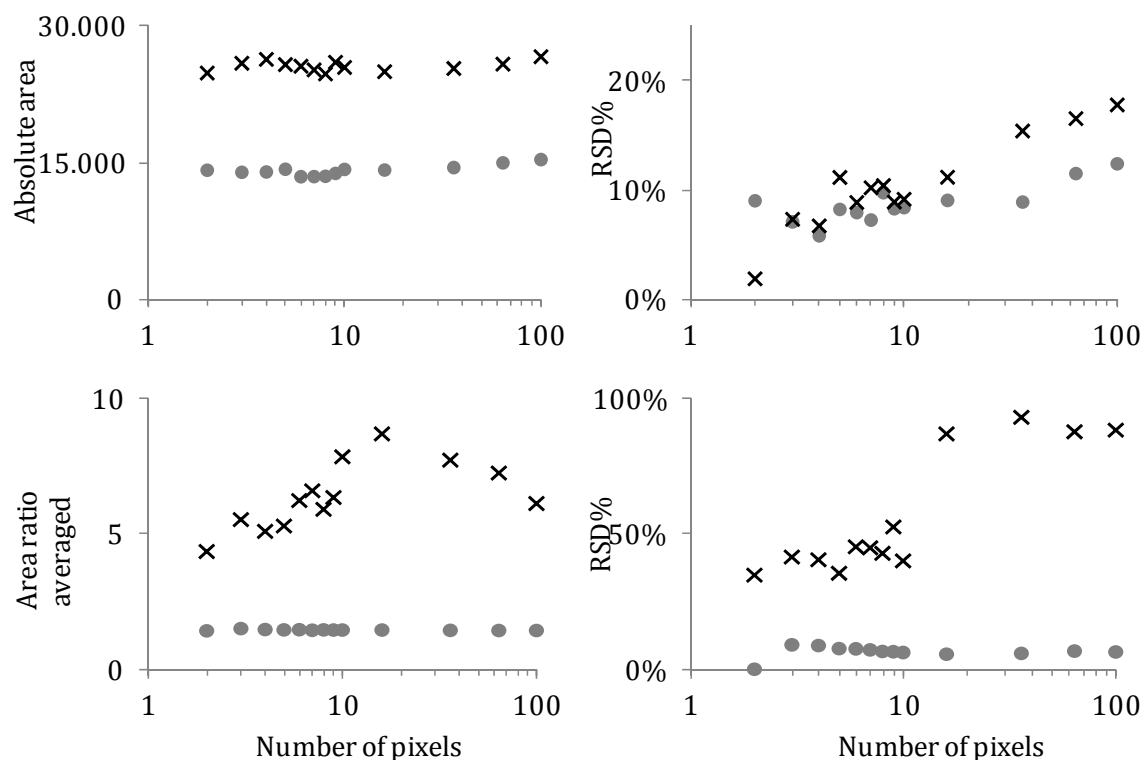


Figure V- 11. (a) Images generated after MALDI-SRM/MSI with experimental parameters set to acquire images with pixel sizes of 50 x 50 μm , 100 x 100 μm and 200 x 200 μm . (b) Images with pixels of 100 x 100 μm and 200 x 200 μm generated after processing of the image with pixels of 50 x 50 μm . Images are displayed after normalization of the absolute signal measured for cocaine by the signal given by cocaine- d_3 .



x Pixels of 100 x 100 μm acquired **•** Pixels of 100 x 100 μm processed

Figure V- 12. Cocaine signal intensity as a function of the number of pixels averaged for pixels of 100 μm x 100 μm . Upper panel: (left) absolute area and (right) corresponding relative standard deviation (RSD in %). Lower panel: (left) absolute area normalized by COC-d₃ and (right) corresponding relative standard deviation (RSD in %). The number of laser shots per pixel is 20.

(x) Pixels of 100 μm x 100 μm acquired as follow: sample stage velocity: 2 mm /s; distance between two raster lines: 100 μm .

(•) Pixels of 100 μm x 100 μm processed from pixels of 50 μm x 50 μm acquired as follow: sample stage velocity: 1 mm/s; distance between two raster lines: 50 μm ; 4 pixels of 50 μm x 50 μm were averaged to generate a pixel of 100 μm x 100 μm in the processed image.

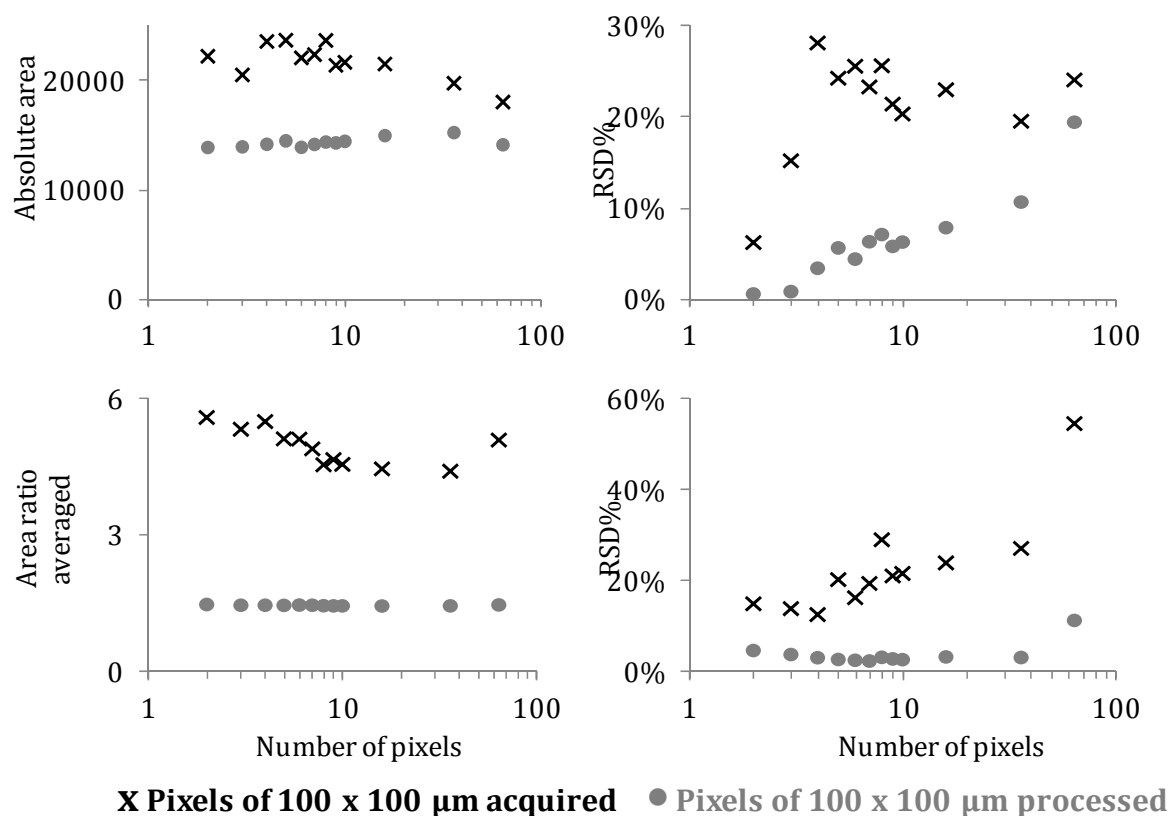


Figure V- 13. Cocaine signal intensity as a function of the number of pixels averaged for pixels of 200 μm x 200 μm . Upper panel: (left) absolute area and (right) corresponding relative standard deviation (RSD in %). Lower panel: (left) absolute area normalized by COC-d₃ and (right) corresponding relative standard deviation (RSD in %).

(x) Pixels of 200 μm x 200 μm acquired as follow: sample stage velocity: 4 mm/s; distance between two raster lines: 200 μm . The number of laser shots per pixel is 20.

(●) Pixels of 200 μm x 200 μm processed from pixels of 50 μm x 50 μm acquired as follow: sample stage velocity: 1 mm/s; distance between two raster lines: 50 μm ; 16 pixels of 50 μm x 50 μm were averaged to generate a pixel of 100 μm x 100 μm in the processed image.

5.2.4. Conclusions

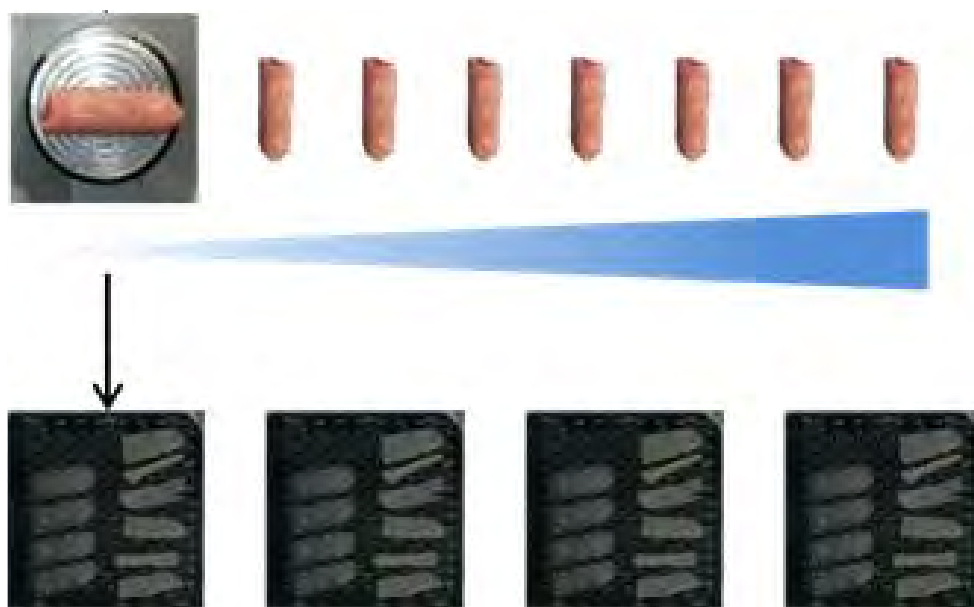
The present work demonstrates that MALDI-SRM/MSI can be quantitative with precision better than 15% if sufficient data are acquired and instrumental parameters correctly set. In a continuous laser raster sampling mode, these instrumental parameters include the sample stage velocity, the distance between two raster lines, the number of laser shots per pixel, the MS duty cycle, the pixel resolution, the laser frequency and the laser energy. Particularly, the latter should be carefully set to avoid snowplow effects which may jeopardize image resolution and quantification. These parameters affect the precision and there is an optimum should be determined for each condition set. It has been shown that a minimum of 3-5 to 10 pixels have to be used to perform quantification with minimum of variability. Drawing region of interest is preferred rather than a comparison pixel-by-pixel (i.e. improved inter-spot variability *versus* intra-spot variability). In general, fast acquisition speed but at low pixel resolution display more variability than low acquisition speed (i.e. with lower pixel size, and thus better image resolution) but is still acceptable and a compromise between analysis time *versus* quality has to be considered/optimized to fit for purpose. Results highlighted the few impact of the sample stage velocity on the variability of the signal measured. On the other hand, the use of high repetition rate laser is mandatory to produce data with good quality, with low spot-to-spot variability (better than 15%) and for increasing the overall signal intensity (see Chapter 3, Part 1, §3.1.2), which correlates previously published data as reported previously in Chapter 1, Part 2 and Chapter 3, Part 1 of this thesis manuscript.

Finally, this work reinforces the fact that internal standard, i.e. a deuterated analog of the analyte herein, is required to improve the data quality and reduce the variability of the signal measured, compared to raw data, and is particularly suited for improving the spot-to-spot variability. The use of deuterated analog is particularly needed in a targeted approach based on a selected reaction monitoring acquisition mode.

CHAPTER 5.

Quantitative MS Imaging (QMSI)

Part 3 - Protocol for the generation of suitable standards for quantification of drugs and their metabolites by (MALDI) MSI - Preliminary results



5.3.1. Introduction

The work presented in this section is based on the latest developments performed within the framework of this thesis, and relies on unpublished data. This protocol has been inspired by Hare *et al.* who previously provided a protocol to produce matrix-matched tissue standards for imaging trace metal (i.e. Cu, Fe, Mg, Mn, Sr and Zn) distribution in brain by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS).¹ The objective of this study is to transfer the method to the analysis of low molecular weight compounds and suggest a comprehensive protocol to generate suitable standards for performing quantitative analysis. The first attempts for generating such standards were made using cocaine and its metabolite benzoylecgonine, spiked at known amount of drugs (i.e. in g per gram of tissue) in bovine kidney, muscle and liver samples. The perspectives of this work are to further utilize these standards in the drug discovery and development process, to link the concentration of a drug within a given specimen to its actual localization. These standards could also be used as references to assess the performances of any imaging method based on MS, such as their limits of detection and quantification, as they already contain a known amount of analyte in g per gram of tissue.

5.3.2. Material and methods

5.3.2.1. Chemicals and reagents

Cocaine (COC), benzoylecgonine (BZE) and cocaine-d₃ (COC-d₃) were provided by Lipomed (Arlesheim, Switzerland). Methanol (MeOH, HPLC grade), ethanol (EtOH, HPLC grade) and acetonitrile (ACN, HPLC grade) were provided by VWR International (Nyon, Switzerland). Formic acid (HCOOH) was provided by Merck (Darmstadt, Switzerland). Harris hematoxylin solution and hydrochloric acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Eukitt(R) quick-hardening mounting medium was provided by Fluka (Buchs, Switzerland). Eosin Y, pure, high purity, biological stain was provided by Acros Organics (New Jersey, USA). Water was purified with a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

5.3.2.2. Tissue samples

Due to the limited amount of postmortem human samples available, the optimization and the preparation of the spiked samples for the calibration were performed using bovine kidney, muscle and liver samples, kindly provided by the Abattoir de Meinier (Meinier, Switzerland).

5.3.2.3. Preparation of spiking solutions

Spiking solutions consisted in 8 solutions of cocaine and benzoylecgonine ranging from 10 to 5'000 ng/mL and prepared in MeOH/H₂O (20/80, v/v). 5 µL of spiking solution were added to 100 mg of tissue samples prior the homogenization (see below). Under these conditions, the final concentration of analytes were of 0.5 – 250 ng per gram of tissue. Standard solution of cocaine-d₃ was prepared in MeOH/H₂O (20/80, v/v) and 5 µL were

either spiked to each aliquot so that the final concentration within the sample is of 50 ng/g of tissue or 1 μ L spotted on the top of the tissue section (i.e. 10 pg spotted) as specified in the text.

5.3.2.4. MALDI matrix

CHCA was used as MALDI matrix and was dissolved at a concentration of 10 mg/mL in appropriate solvent mixture (MeOH:H₂O:HCOOH, 60:40:0.1, v/v/v). 1 μ L of matrix solution was then manually spotted on the top of tissue sections.

5.3.2.5. Homogenization

10 aliquots of 50, 100 or 150 $\pm$ 5 mg of tissue were collected in 10 different Eppendorf tubes of 2 mL and flash frozen in liquid nitrogen (LN₂). Prior the homogenization, a 5 mm-diameter stainless-steel bead was placed in each tube and the tubes were then processed using a Mixer Mill MM400 apparatus (Retsch GmbH, Haan, Germany). Different operating frequencies (i.e. 20, 25 and 30 Hz) were tested over different duration (i.e. 2, 3 and 4 minutes). After homogenization, the 10 aliquots of each sample were centrifuged for 1 minute and the beads were removed using magnets. The aliquots were then flash frozen again and gather in a single tube to form blocks of 0.5, 1.0 or 1.5 g depending on the mass of the initial aliquots (i.e. 50, 100 or 150 mg, respectively). To prepare the spiked tissue, 5 μ L of spiking solution and 5 μ L of cocaine-d₃ solution were added to the aliquots of tissue, prior the freezing in LN₂ and the homogenization steps.

5.3.2.6. Tissue sectioning

Reconstituted blocks were mounted on the sample holder using a droplet of OCT compound (not in contact with the tissue to be further analyzed by MALDI). Frozen samples were brought up to -20°C (i.e. chamber and object temperatures of the cryotome) and cut into 12- μ m sections using a cryotome (Leica Biosystems). Sections were then immediately transferred either on glass slides for microscopy or on stainless steel plates for MALDI mass spectrometry measurements.

5.3.2.7. H&E staining and instrumentation used for microscopy

The complete protocol for Hematoxylin and Eosin (H&E) staining is presented in Supplementary information. Optical images were acquired using a Nikon Eclipse 80i microscope (Nikon Instruments Europe B.V., Switzerland). Images were magnified 20x.

5.3.2.8. MALDI mass spectrometry imaging

MALDI images were acquired in positive ionization mode on a MALDI-4000 QTRAP instrument (AB Sciex) fitted with a frequency-tripled Nd:YAG laser 355 nm (elliptic beam shape of 200 by 100 μ m). Acquisitions were performed in the selected reaction monitoring (SRM) mode. The SRM transitions monitored were: m/z 304 > m/z 182 for cocaine (CE = 27 eV); m/z 290 > m/z 168 for benzoylecgonine (CE = 26 eV) and m/z 307 > m/z 185 for

cocaine-d₃ (CE = 27 eV). The dwell time was set to 28.3 ms for each transition and the total scan time was 100 ms (including 5 ms between 2 transitions). The distance between 2 raster lines was 200 μ m. General operating conditions were: data acquisition mode = rastering; sample stage velocity = 1 mm/s; repetition rate laser = 1'000 Hz; laser energy = 60 μ J; MALDI source and q₀ region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q₂ = 2.4 10^{-5} Torr (nitrogen used as collision gas); declustering potential = 70 V; entrance potential = 10 V; quadrupole resolution was set to unit.

3.3.2.9. Software for data acquisition and processing.

The MALDI source and its laser were controlled using a custom M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and for data collection. A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files (.wiff format) into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex) for SRM/MS images generation and processing. MALDI-SRM/MS images were displayed in TissueView software using the "voxel" mode and the size of each pixel was 200 μ m x 200 μ m. Regions of interest were drawn manually in circles with area varying between 25 and 28 mm² so that the area is smaller than the spot size. Data were exported as text lists and processed in Excel.

5.3.3. Results and discussion

5.3.3.1. Sample preparation workflow

Due to the limited amount of postmortem human samples available, the optimization and preparation of the spiked samples for the calibration were performed using bovine kidney samples. Figure V- 15 illustrates the different steps of the sample preparation followed to generate standards for further quantification by MALDI-MSI. The first step consists of dividing the tissue into 10 aliquots of 50, 100 or 150 $\pm$ 5 mg in 10 different Eppendorf tubes of 2 mL and flash freezing them in liquid nitrogen (LN₂). The reasons why grinding jars with higher size capacity were not used are simple: more material was lost during the grinding step and the size of the final block generated fit perfectly with the size required for samples to be mounted on the sample holder of the cryostat for further sectioning. Using this protocol with 2 mL Eppendorf tubes, more than 95% of the mass of the initial material was recovered.

To prepare the spiked tissue, 5 μ L of spiking solution are added to each aliquot, and a 5 mm-diameter stainless-steel bead is inserted in each tube after being flash-frozen. The tubes are then placed into the Mixer Mill MM400 apparatus (Retsch GmbH, Haan, Germany), designed for dry, wet or cryogenic grinding of small amounts of sample. The homogenization is then performed and different operating frequencies (i.e. 20, 25 and 30 Hz) and duration (i.e. 2, 3 and 4 minutes) were tested. Obviously, increasing the frequency and the time of homogenization is found beneficial to disrupt the biological cells, but also to generate homogenous tissue sections as shown Figure V- 14. After homogenization, the 10 aliquots are centrifuged for 1 minute in and the beads were removed using magnets. The aliquots are

then flash frozen a second time (i.e. as it is easier to take a frozen block off the tube) and gathered in a single tube to form blocks of 0.5, 1.0 or 1.5 g depending on the mass of the initial aliquots (i.e. 50, 100 or 150 mg, respectively). At this stage, it is necessary to thaw the final block, to “stick” aliquots all together

The efficiency of the homogenization in each Eppendorf tube was assessed for several initial amounts of tissue (i.e. 50, 100 or 150 mg) but no significant differences were observed. However, for practical reasons, the optimal condition is set to 100 mg. Indeed, sections cut from a final block of 0.5 g were not large enough to spot several MALDI spots on the top, while 10 aliquots of 150 mg could not be gathered in in single final tube. Finally, the whole protocol is repeated to generate different blocks with different amounts of analyte.

Note that the internal standard (i.e. COC-d3) may be incorporated in the workflow either i) spiked in-tissue at the same time than the analytes are added to the aliquots or ii) spotted on the top of the tissue section prior to MALDI-MS analysis.

The first assays were made by spiking the internal standard in-tissue, to assess if the two analytes plus the internal standard are homogeneously spiked in the tissue, and if it is not the case, if the internal standard can compensate for that heterogenous distribution.

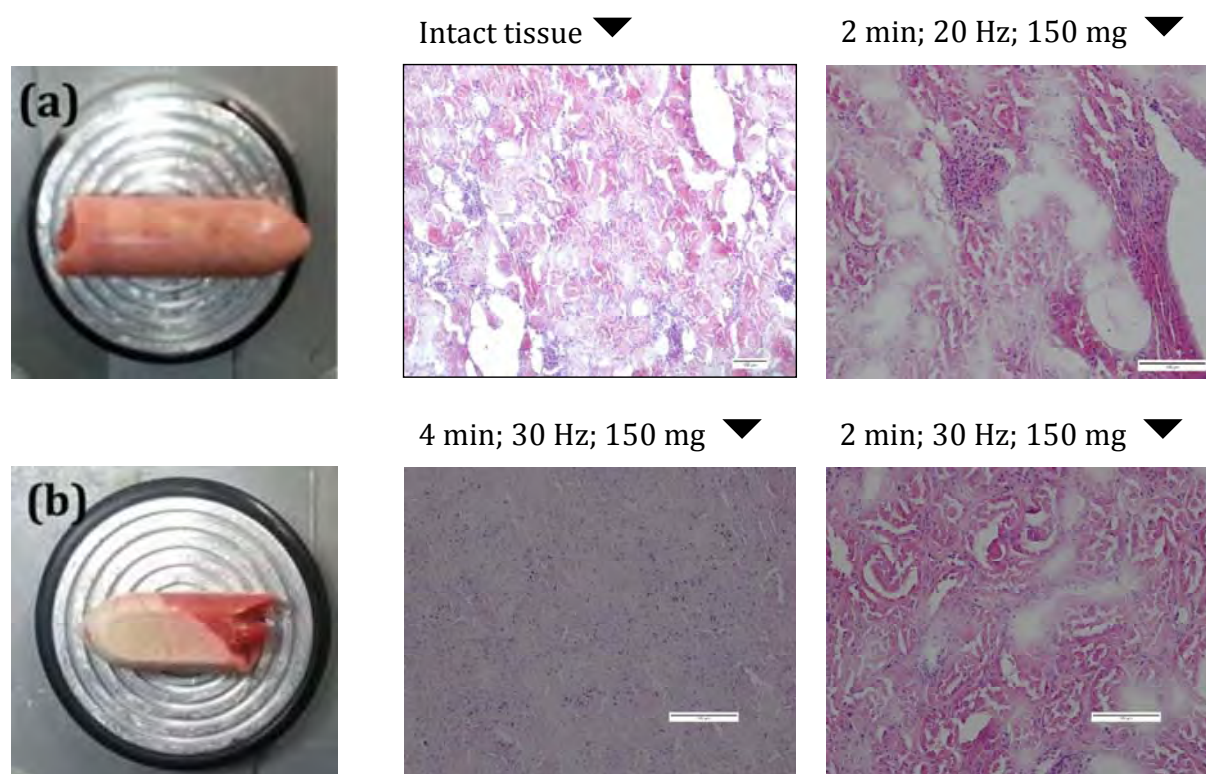


Figure V- 14. Control of tissue sections by microscopy after homogenization/reconstruction of kidney sample. Sections after H&E staining. Optimal conditions of homogenization are 30 Hz for 4 minutes. Final block after homogenization/reconstruction of bovine (a) kidney and (b) muscle samples.

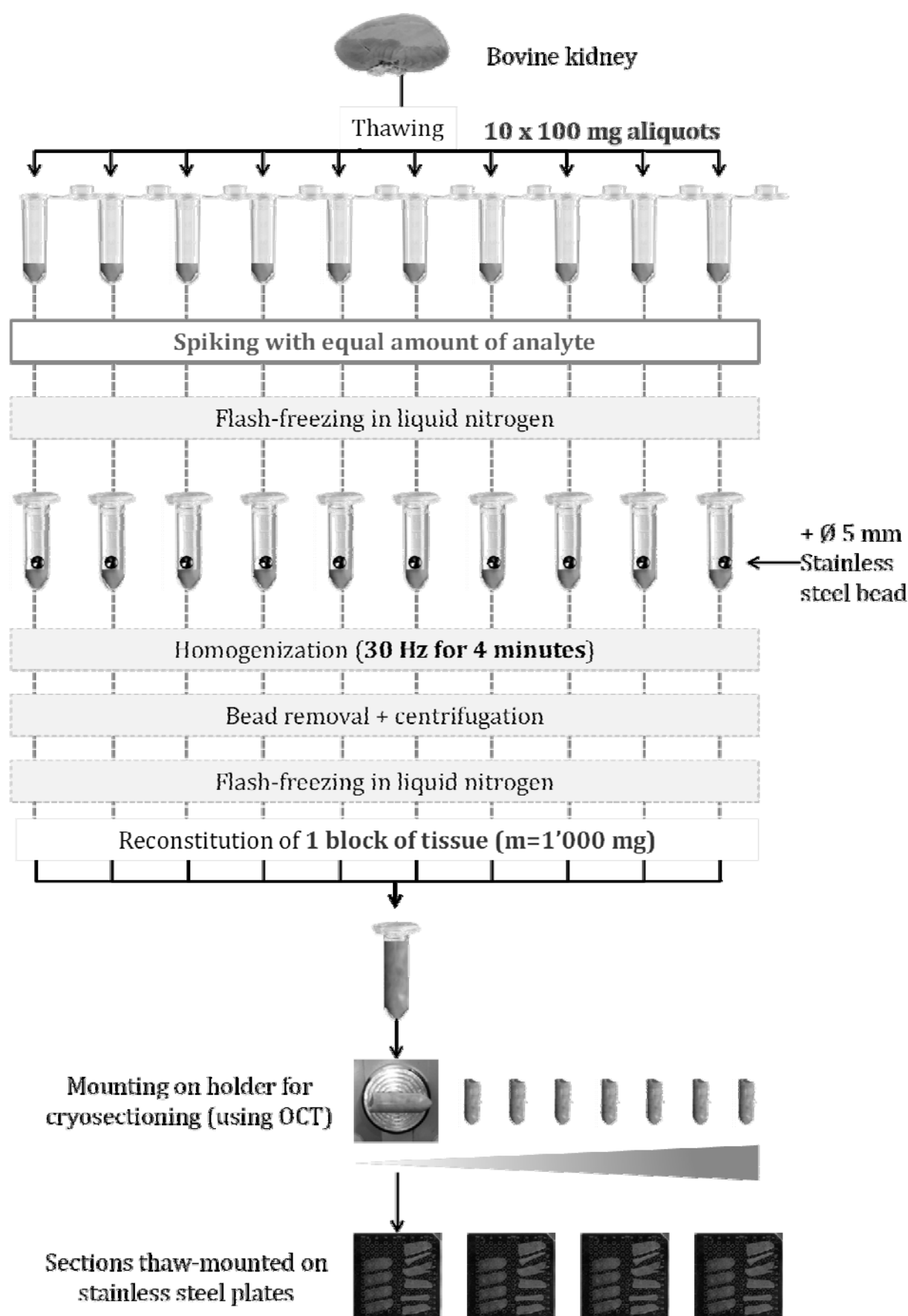


Figure V- 15. Protocol established to generate standards following the "in-tissue" strategy. 10 aliquots of tissue are independently spiked with equal amount of analyte and homogenized, and finally gather to form a reconstructed block. The protocol is repeated to generate different block with increasing concentration of drugs.

5.3.3.2. Assessment of the homogenous distribution of the analytes within the tissue section by MALDI-MSI

a) Variability in function of the type of tissue

To assess the homogeneity of the distribution of the analyte within the spiked tissue, tissue sections were analyzed by MALDI-MS imaging. The first observation is the inter-tissue variability (Figure V- 16), which was expected due to tissue-specific ion suppression effects.

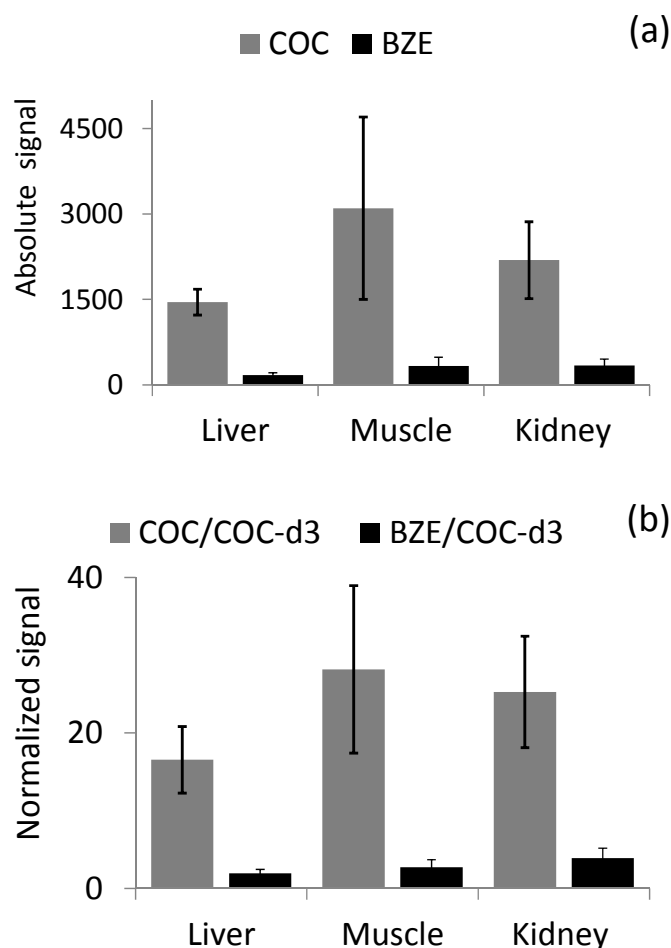


Figure V- 16. (a) Absolute and (b) normalized signals measured for COC and BZE after MALDI-SRM/MSI analysis of tissue sections sectioned after homogenization/reconstruction of liver, muscle and kidney samples. Error bars represent ± 1 standard deviation calculated on the averaged signals measured for 3 spots for all tissue sections ($n=12$ for liver; $n=15$ for muscle and $n=21$ for kidney). Normalized signals are given after normalization by COC-d₃.

Then, the intra- and inter- section variability are given Table V- 7. Intra- and inter-section variability measured for cocaine and benzoylecgonine after MALDI-SRM/MSI analysis of tissue sections sectioned after homogenization/reconstruction of liver, muscle and kidney samples. The intra-section relative standard deviations (RSD, in %) are given for: $n=4$ for liver; $n=5$ for muscle and $n=7$ for kidney. The inter-section RSD (in %) is given for: $n=12$ for liver; $n=15$ for muscle and $n=21$ for kidney. Table V- 7. Intra-section variability is usually lower than the inter-section variability. The highest variability was measured for both analyte in muscle, which can be easily correlated to the aspect of the final block of tissue, as shown Figure V- 14.b, compared to the homogenous tissue reconstructed from kidney

homogenates (Figure V- 14.a). The overall variation measured in liver is about 20-25% and above while the variation in kidney is around 30%. This emphasizes again the fact that such an approach can be applied to one type of tissue only, and that the protocol has to be optimized for each.

It is worth noting that the addition of internal standard in the tissue does not contribute to improve the variability. This leads to the conclusion that the different compounds are not incorporated homogeneously or in the same manner into the tissue, and this way to incorporate the internal standard is not appropriate to compensate such variability.

Table V- 7. Intra- and inter-section variability measured for cocaine and benzoylecgonine after MALDI-SRM/MSI analysis of tissue sections sectioned after homogenization/reconstruction of liver, muscle and kidney samples. The intra-section relative standard deviations (RSD, in %) are given for: n=4 for liver; n=5 for muscle and n=7 for kidney. The inter-section RSD (in %) is given for: n=12 for liver; n=15 for muscle and n=21 for kidney.

Tissue	# of sections	Intra-section variation (RSD,%)				Inter-section variation (RSD,%)			
		COC	COC/	BZE	BZE/	COC	COC/	BZE	BZE/
			COC-d ₃		COC-d ₃		COC-d ₃		COC-d ₃
Liver	4	1 to 18%	32% to 99%	8 to 27%	13 to 27%	16%	26%	23%	26%
		(10%)	(43%)	(18%)	(20%)				
Muscle	5	7 to 46%	9 to 32%	8 to 44%	8 to 44%	52%	38%	46%	36%
		(27%)	(25%)	(29%)	(30%)				
Kidney	7	20 to 36%	15 to 30%	12 to 42%	13 to 36%	31%	28%	32%	33%
		(29%)	(24%)	(29%)	(26%)				

As the objective the first objective of this study is to apply the methodology to the quantification of drugs of abuse in human postmortem samples, for forensics purpose, and that only kidney samples are available, further assays will be performed on kidneys sample only.

b) Generation of “in-tissue” dilution range

A series of spiked tissues containing both COC and BZE at concentration ranging from 0.5 to 250 ng/g and equal amount of COC-d₃ at a 50 ng/g of tissue are generated and sectioned into sections of 12 µm-thicknesses. The resulting MALDI-SRM/MS images are displayed Figure V- 17 and Figure S1 and the corresponding quantitative results are presented Table V- 8.

Although the analytes could be detected with a signal-to-noise ratio above 3 even at the lowest level of concentration ranging from 0.5 to 250 ng_{analyte}/g_{tissue} the performances in term of precision and accuracy (re-calculated from the equation of the linear regression and from the same spots measured to build the curve, i.e. “pre-validation” of the method) could not be detected because of too high variability measured (Table S1) and the precision and accuracy values were >> 40-50%.

To obtain acceptable accuracies within 88-125% (Table V- 8), the dilution range considered was 25-250 ng_{analyte}/g_{tissue}. But still the precisions were out of the acceptable limits in the most of the case (i.e. 11-41%). There is no doubt that further developments are needed to improve the strategy.

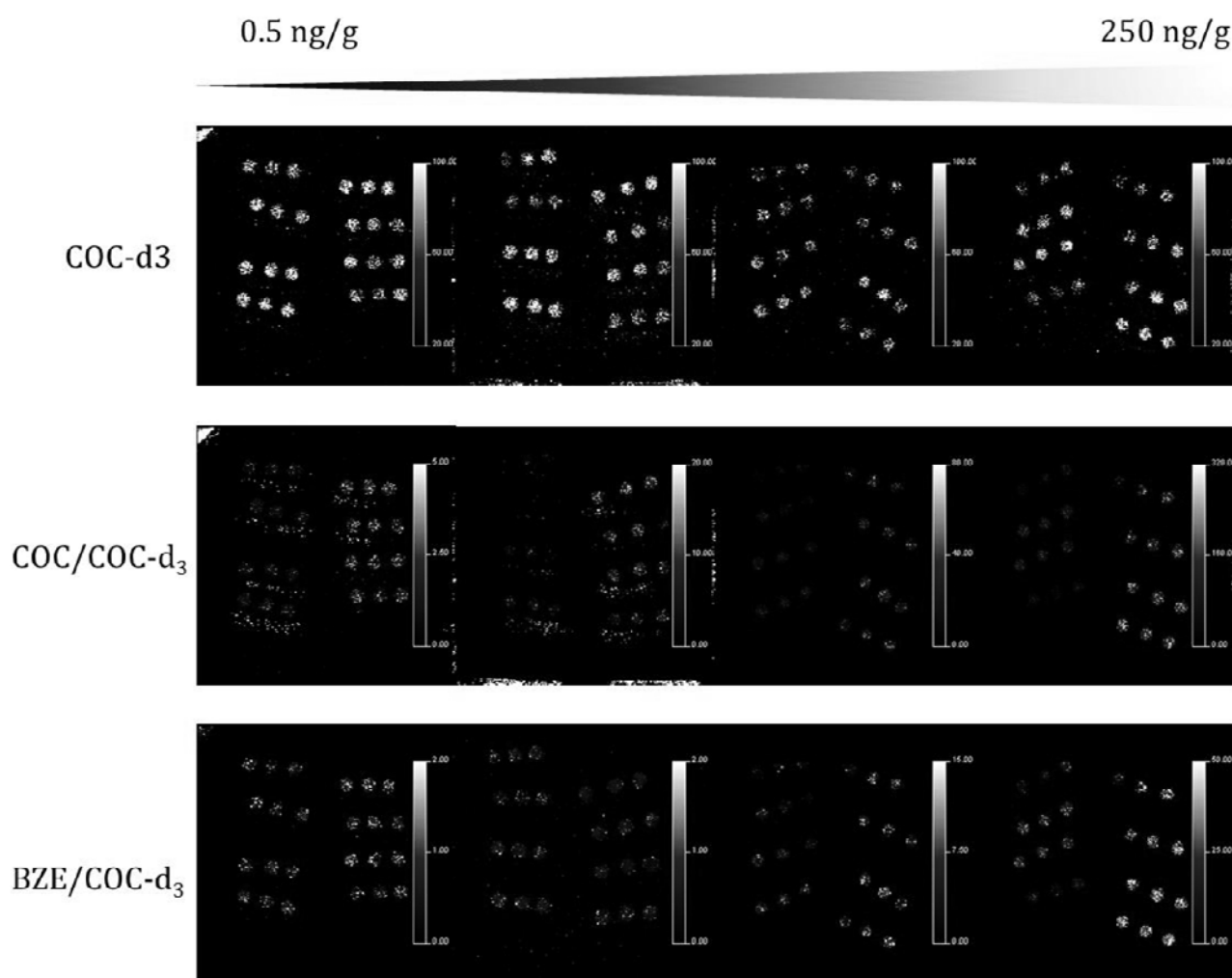


Figure V- 17. MALDI-SRM/MS images based on the SRM traces for COC-d₃, COC and BZE. The image for COC and BZE are displayed after normalization by the signal measured for COC-d₃. Four sections per tissue spiked at a given level of concentration were mounted onto the plate. From left to right: 0.5; 2.5; 5; 12.5; 25; 50; 125 and 250 ng/g of reconstructed kidney. The vertical color scale represents the respective SRM signal intensity of the analyte normalized by COC-d₃ (absolute signal for COC-d₃): white pixels give the highest signal; dark pixels give the lowest signal.

Table V- 8. Quantitative MALDI-SRM/MS imaging results: precision and accuracy calculated for cocaine and benzoylecgonine from in-tissue dilution range. COC-d₃ was spiked into the tissue samples at the same time than the analyters. Concentrations are given in ng_{analyte}/g_{tissue}. Three spots were measured per section and the recalculated concentration displayed in the table is based on the averages of measurement of the 3 spots. The accuracy and the precision are calculated for n=4 (i.e. for 4 tissue sections).

(a)	Expected conc.(ng/g)	Recalculated concentration (per section, ng/g)					Mean	Accuracy	Precision
Cocaine	25.0	24.8	29.1	33.3	37.9	31.3		125%	18%
	50.0	42.6	45.6	60.8	60.8	52.5		105%	19%
	125.0	77.1	139.8	143.5	78.5	109.7		88%	34%
	250.0	166.0	215.4	322.3	322.3	256.5		103%	31%
	Slope	41.41							
	Intercept	-635.30							
	R2	0.83							
(b)	Expected conc.(ng/g)	Recalculated concentration (per section, ng/g)					Mean	Accuracy	Precision
COC/COC-d ₃	25.0	20.8	26.8	33.0	40.7	30.3		121%	28%
	50.0	43.7	47.8	54.9	54.9	50.3		101%	11%
	125.0	83.8	152.2	151.0	72.3	114.8		92%	37%
	250.0	175.0	246.1	276.1	320.6	254.5		102%	24%
	Slope	0.47							
	Intercept	-5.73							
	R2	0.87							
(c)	Expected conc.(ng/g)	Recalculated concentration (per section, ng/g)					Mean	Accuracy	Precision
BZE	25.0	23.5	27.6	32.8	42.7	31.6		127%	26%
	50.0	44.2	43.7	64.5	64.5	54.2		108%	22%
	125.0	73.8	141.1	143.3	67.1	106.3		85%	39%
	250.0	162.7	221.2	331.2	316.3	257.8		103%	31%
	Slope	11.45							
	Intercept	-156.43							
	R2	0.82							
(d)	Expected conc.(ng/g)	Recalculated concentration (per section, ng/g)					Mean	Accuracy	Precision
BZE/COC-d ₃	25.0	19.9	24.7	31.0	46.6	30.5		122%	38%
	50.0	46.0	45.1	57.5	57.5	51.5		103%	13%
	125.0	82.1	152.4	152.6	63.5	112.6		90%	41%
	250.0	177.9	254.0	278.6	310.9	255.3		102%	22%
	Slope	0.13							
	Intercept	-1.12							
	R2	0.87							

5.3.4. Conclusion and perspectives

This objective of this work was to establish a protocol to generate standards for quantitative MS imaging, based on the “in-tissue” dilution range strategy. The main advantage of this method is to take into account the matrix effect specific to the tissue considered. Still, this method cannot be extended to other organ and sample preparation has to be optimized for each type of tissue.

The results obtained in terms of accuracy and precision are not the ones expected and need further dramatic improvements to attain performances better than 15%, achieved by LC-ESI-MS/MS or in “conventional” MALDI-MS/MS analysis.

Several attempts have been made to reduce the intra- and inter-section variability of the signal measured for the analytes, such as reducing the amount of organic solvent used to dissolve the standard. This allows reducing the intra-section variability from 25-30% to < 20% (Figure S2, Table S2). This makes sense considering the amount of water containing in a tissue such as kidney. However, it does not contribute to reduce the inter-section variability

A comparison between two strategies to incorporate the internal standard, i.e. spiking versus spotting, displays an improvement of the intra- and inter-section variability below 20% (Figure S3, Table S3), with and without normalization. The latter result is encouraging since, considering the final application, the internal standard will be spotted and cannot be spiked into the forensic sample, of which integrity will be maintained intact as much as (i.e. the “real sample will only be sectioned and analyzed without any further modifications). Variability below 20% match with the performance we can expect from MALDI-SRM/MSI, according to the conclusion made Chapter 5, Part2.

Increasing the duration of the homogenization could also be an option (i.e. the objective during the method development was to reduce sample handling, but this has to be reconsidered). Another modification of the sample preparation will be the application of the matrix: an automated spraying will be performed to cover homogeneously the surface of the tissue section, in order to reduce possible variation caused by manual spotting.

Finally, and interestingly, there were no significant differences between the results displayed with or without normalization of the signals by the signal of deuterated cocaine. However, further investigations are needed to state about the relevance of using an internal standard in the present approach.

5.3.5. Supplementary material

Protocol: Hematoxylin and Eosin (H&E) staining

This staining protocol combines a nuclear staining, thanks to the Harris hematoxylin, and a staining of cytoplasm and collagen thanks to Eosin Y.

As a result,

- o **Nuclei** appear blue-violet (dark)
- o **Cytoplasm** are pink
- o **Erythrocytes** are red (light) to yellow
- o **Collagen fibers** are pink-orange (depending on the acidophilic properties of different elements)

This protocol has been adapted from <http://www.bcm.edu/rosenlab> for H&E staining for frozen sections. Some cautions have to be taken to section / mount the frozen sections. All the specific material is indicated below.

Material:

- Harris hematoxylin solution, modified (Sigma Aldrich, Part. Number HHS16, 500 mL)
- Eosin Y (yellowish) (Acros organics, Part Number 15288, 100 gr): prepare 1% of Eosin Y (1 g for 100 mL)
- Xylene (laboratory grade)
- Eukitt quick hardening mounting medium for microscopy (Fluka, Part Number 03989, 100 mL)
- Acidic alcohol: 1% hydrochloric acid in 70% ethanol (EtOH)

Sample preparation:

1) Preparation of tissue sections:

- o Freezing tissue for cryostat sectioning: tissue should preferably be snap-frozen by quenching in liquid nitrogen (except for muscle: use cold isopentane [2-methylbutane] or crystal artifact will occur)
- o Attached the tissue sample by putting a small amount of OCT compound onto a cork disc and leave in the cryostat for some minutes to freeze it (OCT starts to freeze below -10°C)
- o Cryosectioning of frozen samples at
- o Make sections of 10-12 μm thickness
- o Mount onto SuperFrost

2) Hematoxylin staining:

/!\ Caution: Skim surface of hematoxylin with a Kimwipe to remove oxidized particles. Blot excess water from slide holder before going into hematoxylin

- o 1 x 3 minutes Hematoxylin (staining)
- o Rinse Wash in deionized water
- o 1 x 5 minutes Tap water (to allow stain to develop)
- o Dip 8-12X (fast) Differentiate in Acid alcohol: 1% hydrochloric acid in 70% EtOH
- o Rinse 2 x 1 minute Tap water
- o Rinse 1 x 2 minutes Deionized water (*can leave overnight at this stage*)

3) Eosin staining and dehydration

/!\ Caution: Blot excess water from slide holder before going into eosin

- o 1 x 30 seconds Eosin 1% (*up to 45 seconds for an old batch of eosin*)
- o 3 x 5 minutes 95% ethanol
- o 3 x 5 minutes 100 % ethanol (*blot excess ethanol before going into xylene*)
- o 3 x 15 minutes Xylene

Total time of sample preparation until this step = ~1h30

4) Coverslip slides using the mounting medium

- o Mounting medium used: Eukitt quick-hardening mounting medium (Fluka, Part. Number 03989)
- o Place a drop of Eukitt on the glass slide using a glass rod, taking care to leave no bubbles
- o Angle the coverslip and let fall gently onto the slide. Allow the Eukitt to spread beneath the coverslip, covering all the tissue

5) Dry overnight in the hood.

Table S1. Intra- and inter-section variability measured for COC and BZE after MALDI-SRM/MSI analysis of tissue sections generated for the in-tissue dilution range. Values in brackets display the averaged variability for the 4 sections (3 spots were measured per section); thus the intra-section variability is given for n=4.

Conc. ng/g	Intra-section variation (RSD,%)				Inter-section variation (RSD,%)			
	COC	COC/ COC-d ₃	BZE	BZE/ COC-d ₃	COC	COC/ COC- d ₃	BZE	BZE/ COC- d ₃
0.5	7 to 32% (14%)	4 to 32% (18%)	12 to 26% (18%)	9 to 22% (17%)	23%	20%	19%	19%
2.5	14 to 34% (24%)	5 to 22% (14%)	1 to 49% (14%)	6 to 31% (19%)	27%	20%	30%	25%
5	16 to 50% (27%)	38 to 173% (83%)	8 to 47% (83%)	6 to 21% (13%)	42%	82%	52%	18%
12.5	12 to 29% (21%)	8 to 65% (36%)	14 to 46% (36%)	5 to 30% (17%)	33%	43%	41%	20%
25	8 to 48% (20%)	5 to 65% (28%)	16 to 55% (28%)	13 to 65% (30%)	37%	49%	49%	54%
50	6 to 39% (17%)	16 to 26% (21%)	8 to 49% (21%)	20 to 50% (33%)	35%	24%	45%	36%
125	7 to 27% (16%)	1 to 49% (22%)	5 to 39% (22%)	5 to 48% (23%)	37%	41%	43%	43%
250	6 to 47% (27%)	8 to 69% (33%)	10 to 53% (33%)	10 to 65% (35%)	37%	35%	40%	36%

Table S2. Intra- and inter-section variability measured for COC and BZE to assess the effect of the solvent used to dissolve the standard on the variability of the signal. The percentage of MeOH represents the proportion of organic solvent used to dissolve the standards to prepare spiking solutions. Values in brackets display the averaged variability for the 4 sections (3 spots were measured per section).

%MeOH	Intra-section variation (RSD,%)				Inter-section variation (RSD,%)			
	COC	COC/ COC-d ₃	BZE	BZE/ COC-d ₃	COC	COC/ COC-d ₃	BZE	BZE/ COC-d ₃
20%	20 to 36% (29%, n=4)	15 to 30% (24%, n=7)	12 to 42% (29%, n=7)	13 to 36% (26%, n=7)	31%	28%	32%	33%
0%	6 to 22% (16%, n=4)	9 to 26% (17%, n=4)	12 to 26% (19%, n=4)	3 to 28% (16%, n=4)	36%	19%	33%	21%

Table S3. Intra- and inter-section variability measured for COC and BZE to compare the influence of spiking or spotting the internal standard. H₂O is used as solvent to prepare the spiking solutions. Values in brackets display the averaged variability for the 4 sections (3 spots were measured per section).

COC-d ₃	Intra-section variation (RSD,%)				Inter-section variation (RSD,%)			
	COC	COC/ COC-d ₃	BZE	BZE/ COC-d ₃	COC	COC/ COC-d ₃	BZE	BZE/ COC-d ₃
Spiked	6 to 22% (16%, n=4)	9 to 26% (17%, n=4)	12 to 26% (19%, n=4)	3 to 28% (16%, n=4)	36%	19%	33%	21%
Spotted	4 to 28% (13%, n=4)	13 to 26% (18%, n=4)	2 to 15% (9%, n=4)	2 to 13% (7%, n=4)	17%	16%	17%	10%

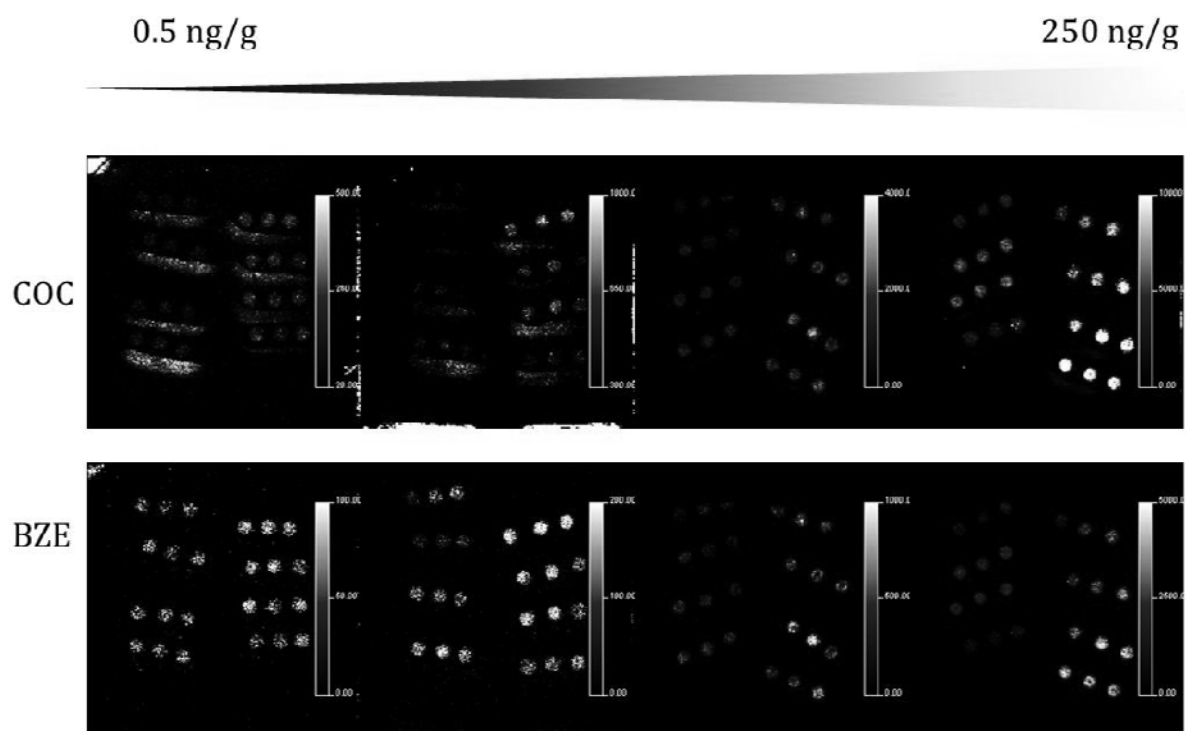


Figure S1. MALDI-SRM/MS images based on the SRM traces for COC and BZE without normalization. Four sections per tissue spiked at a given level of concentration were mounted onto the plate. From left to right: 0.5; 2.5; 5; 12.5; 25; 50; 125 and 250 ng/g of reconstructed kidney. The vertical color scale represents the respective SRM signal intensity of the analyte: white pixels give the highest signal; dark pixels give the lowest signal.

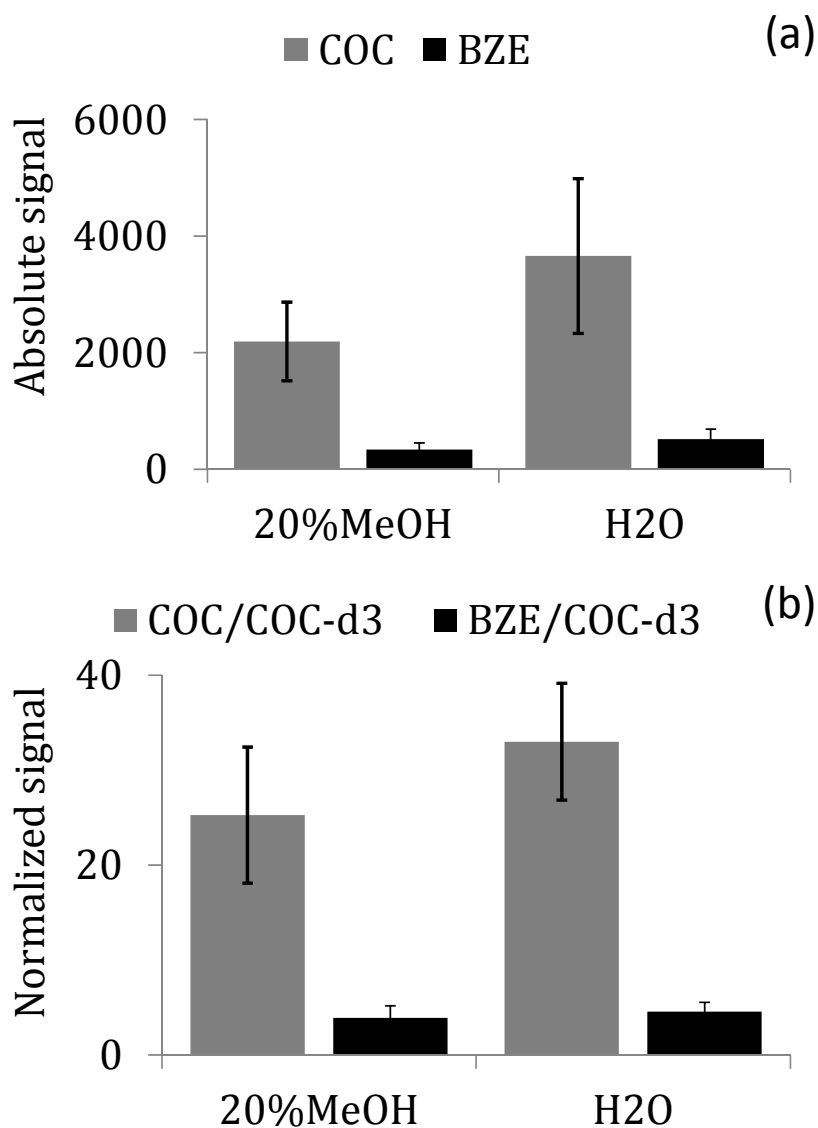


Figure S2. Effect of the solvent used to dissolve the standard on the variability of the signal. (a) Absolute signals measured for COC and BZE. (b) Signal given after normalization by COC-d₃ for COC and BZE. The concentration for COC and BZE was 10 ng/g and COC-d₃ was spiked into the tissue at a concentration of 50 ng/g.

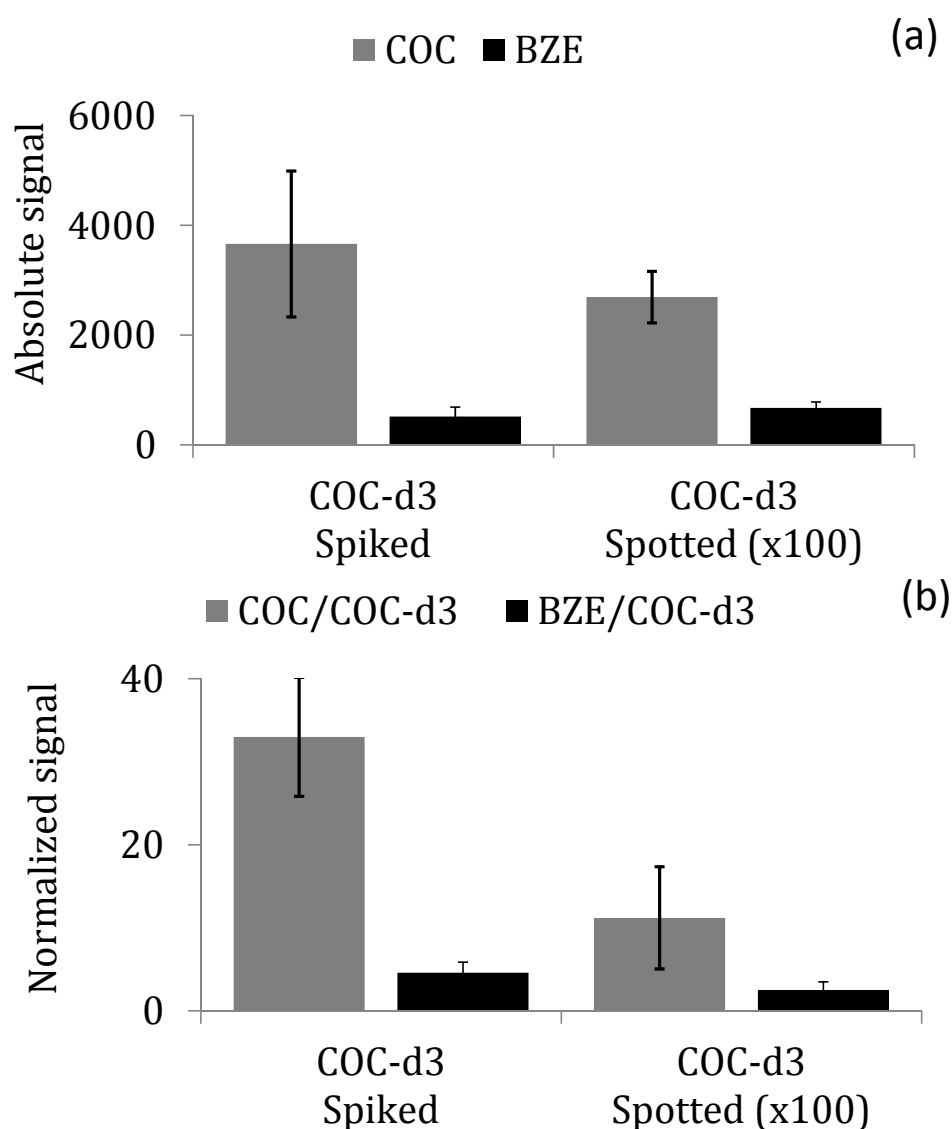


Figure S3. Effect of the way to incorporate the internal standard on the variability of the signals measured. (a) Absolute signals measured for COC and BZE. (b) Signal given after normalization by COC-d₃ for COC and BZE. The signals obtained after spotting COC-d₃ on the top of the tissue section are multiplied by 100. The concentration for COC and BZE was 10 ng/g and COC-d₃ was spiked into the tissue at a concentration of 50 ng/g. For the spotting condition, every spot measured contained 10 ng of COC-d₃ (i.e. 1 μ L of a solution at 10 ng/ μ L was spotted onto the sections).

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GENERAL CONCLUSIONS AND PERSPECTIVES

Although some perspectives have already been explored through this thesis manuscript, this general conclusion will summarize them, and propose some avenues for future development.

Is there a future for MSI?

The beginning of this thesis highlighted the power of mass spectrometry imaging in biomedical imaging, in particular in drug discovery and development and also in biomarker discovery directly from complex biological samples. By providing chemical specificity via the analyte's mass and localization without any prior knowledge of the imaging target, MSI allows not only for targeted analysis but also for the discovery of unknown species. The ability to distinguish a parent drug administered to its metabolites and accurately determine the spatial localization of both analytes is a strength of MSI. The developments that have been achieved in pushing forward the limits in terms of ultra-high spatial resolution, high throughput, and high sensitivity offer a large panel of analytical possibilities to answer biologically relevant questions, for a wide variety of biological samples and molecular species, from small molecules to high mass proteins. Nowadays, researchers can make use of high mass resolution, high mass accuracy, tandem MS strategies or even ion mobility to identify molecular species detected on biomedical tissue surfaces. These tools help to better understand the underlying biology of health and diseases and to improve treatments.

Therefore, there is no doubt that mass spectrometry imaging will continue to expand in the future.

Major achievements of this thesis

The major objective of this thesis was to continue to spread the use of MSI and develop applications to the analysis of drugs and their metabolites in plant biology and forensic toxicology. Thus, two platforms based on triple quadrupole linear ion trap mass analyzers (QTRAP) were used.

The first platform consists of a QTRAP fitted with a high repetition rate matrix-assisted laser desorption/ionization (MALDI) source, operated at intermediate pressure; the platform has been utilized for its ability to generate and acquire data in a rapid fashion, for its high sensitivity and selectivity, provided by the selected reaction monitoring (SRM) and enhanced product ion scan (EPI) modes. The second platform makes use of two technologies: a chip-based nano-electrospray ionization source operated under ambient conditions capable of liquid extraction surface analysis (LESA) and differential ion mobility mass spectrometry (DMS), allowing the gas-phase separation of isomers prior to MS detection.

The MALDI-SRM/MS platform has been used as part of the study of the evolution of tropane alkaloid biosynthesis in *Erythroxylum coca* plants. MALDI-SRM/MSI demonstrated the even distribution pattern across the leaf in *E. coca* of tropane alkaloids such as cocaine,

cinnamoylcocaine and ecgonine methyl. Furthermore, the platform provided structural confirmation of these compounds by recording their respective MS/MS spectra, at three different stages of the development of the plant (i.e. early stage, intermediate stage referring the expanding of the leaves and mature stage). A straightforward and rapid workflow has also been proposed to monitor drugs and their metabolites in intact hair samples for forensic investigations. Thus, MALDI-SRM/MSI provides useful information about chronological consumption of cocaine contained in a single intact hair over several months in less than 10 minutes. Very few publications report the application of MSI to postmortem human tissue, and this work has proven the capability of MALDI-SRM/MSI to easily perform rapid screenings in tissue sections combined with confirmatory MS/MS analysis to prove illicit drugs consumption. However, the main limitation of such direct surface analysis approaches remains the analysis of isomers and isobaric compounds, and this is where the LESA-DMS platform plays a key role.

Multimodality imaging

One of the research areas that has benefited from particular attention is the development of ionization sources, and this thesis highlighted the importance of multimodality imaging to answer a particular biological question, and presented three main approaches, namely SIMS, MALDI and LESA, each with their own strengths and limitations.

SIMS is undeniably the favorite technique of high spatial resolution imaging and showed unequalled performance in this domain so far. Within the framework of this thesis, ToF-SIMS has been identified as a powerful tool to support deeper investigation of biological mechanisms of drug incorporation into hair and provided valuable information on the distribution of cocaine and its metabolites within the different layers of the hair architecture (work which has never been described by other working groups). Then, ToF-SIMS also generated promising results for the analysis of *E. coca* leaves cross-sections at the mature stage. Preliminary results correlate well with previous immunolabeling assays and allow localizing cocaine and ecgonine methyl ester in the upper epidermis and mesophyll in a leaf at the mature stage of development, which was not possible with MALDI-MSI on intact leaves due to important matrix effects (see below). For both hair and leaf sample analysis, the main challenge remains sample preparation prior to MS analysis. Thin intact hair is difficult to handle and to cut manually; and leaves are rather fragile samples, in that they are easily breakable. In addition, due to extensive fragmentation caused by the primary ion beam in SIMS, a significant lack of sensitivity is visible compared to MALDI (i.e. for the hair study: only COC and ECOC metabolites could be detected by SIMS versus 3 by MALDI [i.e. MALDI could also detect BZE/NCOC]; for the leaf study: only COC and EME could be detected while MALDI also enables the detection of cinCOC. Finally, the ToF-SIMS platform suffered from the absence of identification capabilities, possible with the MALDI-MS platform with

MS/MS. The hyphenation of SIMS with triple quadrupole mass analyzers could be one of the next developments for this method.

This thesis emphasizes the performance of the **MALDI-QTRAP** platform for fast targeted screening and MS/MS confirmatory analysis of drugs and their metabolites from different types of tissue, namely plant leaves, intact hair and postmortem tissue sections. It also highlights the main limitations of the platform, i.e. the spatial resolution achieved in the current instrumental setup and the lack of selectivity for the analysis of isomeric compounds, which could be complemented by ToF-SIMS and LESA-DMS-MS/MS, respectively. Despite these limitations, plus the fact that MALDI encounters some critical limitations due to the application of the matrix, it remains the most widely used ionization technique in MS imaging and developments are still carried out to overcome these barriers.

The last few years have also seen the emerging field of ambient ionization techniques broaden the analytical possibilities. Among these techniques, liquid extraction surface analysis (**LESA**) has been demonstrated complementary to MALDI, and offers the non-negligible advantage to require quasi no sample preparation prior to the analysis. Also, LESA has some limitations and is not fully recognized as an imaging modality due to its lack of spatial resolution, and because most of the results are not associated with MS images. However, here again, with the objective to develop the LESA approach, several relevant strategies have been proposed, i.e. to improve the desorption efficiency, the spatial resolution and for use in combination with orthogonal techniques to allow separation of isobaric species (see perspectives Chapter 4, Part 2 for more details). In addition, new software has been recently introduced and allows for the generation of images, where each discrete spot analyzed by LESA could be displayed along with its intensity. Although very few publications report the use of LESA in drug discovery and development, one can expect the LESA (DMS) platform to continue to develop and further development will contribute to its full recognition in the MS imaging field as an essential simple and rapid analytical tool for direct tissue profiling. In addition, one of the benefits of LESA is the long and stable spray generated after extraction of analyte from the sample and thus LESA is ideally suited for prolonged MS/MS analysis. In this manuscript, information-dependent acquisition (IDA), or dependent-data acquisition (DDA), was presented as particularly adapted for the screening and confirmation of drugs of abuse extracted from postmortem tissue sections, but limitations for low abundant precursor ions can be neglected. Recently, qualitative and quantitative workflows based on data-independent approaches, namely MS^{ALL} (or MS^E) (i.e. collecting TOF mass spectra with and without fragmentation by alternating the collision energy of the collision cell between low and high values) or sequential window acquisition of all theoretical fragment-ion spectra (SWATH), were developed on hybrid QqTOF mass analyzers (that offer resolving power 20,000-40,000). These approaches were

demonstrated to be particularly powerful in detecting low abundant analytes for further metabolite and peptides identifications.^{1,2} LESA is a perfect candidate for such hyphenation and future applications in metabolomics or proteomics are very likely.

Ion mobility

The interest in ion mobility in MS imaging has considerably increased over the last few years and this thesis emphasizes the benefits to add an additional separation based on ion mobility after direct surface analysis, such as DMS. Because DMS operates under ambient conditions, one can expect to hyphenate this technology after any kind of ionization source operated at atmospheric pressure.

In this thesis, DMS, assisted with an organic modifier, is presented as a sensitive tool used together with LESA to investigate drugs of abuse and metabolites in postmortem forensic samples, down to the ng/g range. Although the differential mobility behavior of analytes cannot be clearly anticipated, DMS can act as a chromatographic separation for ions in the gas-phase and is demonstrated to considerably improve the selectivity of a conventional surface sampling-based platform operated at atmospheric pressure prior to MS detection. So far, and in the current instrumental setup developed, the main limitation of the speed of acquisition is the MS duty cycle. Even with the SRM mode, which is relatively fast compared to other acquisition modes, the duty cycle increases with the number of transitions sequentially monitored and can be in the order of a second. This can be considerably improved, however, by the latest generation of QqTOF-based mass spectrometers where all the ions are collected in parallel to generate one full spectrum in 10 ms. The combination of LESA-DMS-QqTOF is also one of the innovative platforms expected in the near future to broaden perspectives in many fields, including metabolomics and proteomics.

Quantification by MSI: to each sample, its own matrix effect

As discussed in the last Chapter of this thesis, quantification by mass spectrometry imaging is by far the hottest topic in current discussions by experts in the field, and collective efforts are put together to demonstrate the quantitative ability of MS-based imaging approaches. Different approaches, labeled or label-free, have been developed for QMSI for endogenous and/or exogenous compounds, from small drugs to peptides or proteins. Most of them were proven robust in term of linearity and quantitative results generally correlate well with LC-MS of tissue homogenates. However, the quest for the perfect dilution series to better mimic the behavior of an analyte in its biological environment is still challenging, and has to consider several fundamental aspects inherent to the analysis of complex biomatrices such as (animal) **tissues**. These aspects include tissue- and analyte- specific ion suppression effects, analyte extraction efficiency from the tissue sections, and diffusion of the analyte standards within the tissue when used. Most of these approaches may fulfill some of these limitations but rarely all of them. Some attempts have been made within the framework of

this thesis, and although the results were not the ones expected so far, there are some perspectives that hint at success.

Throughout this research, the tissue-specificity of ion suppression effects has been emphasized as the most challenging issue in quantification by MSI. In this respect, each type of tissue has to be considered independently, and methods optimized for each.

For the study of **intact leaves**, two key parameters affected quantitative performance of the MALDI-SMR/MSI approach, even for relative quantification:

i) the matrix effect, due to different waxy cuticle composition and thickness between upper and lower epidermis; the upper surface of coca leaves has a thick waxy cuticle rich in lipids that may be responsible for important suppression effects decreasing the response of ions corresponding to tropane alkaloids present within the leaf;

and ii) analyte extraction efficiency. This effect is more pronounced in intact leaves as the analytes have to be extracted from deeper layers, composed by a heterogeneous population of cells. This is in contrast with animal tissue sections, where cells are supposed to be “opened”, and thus are easily accessible by the ionizing laser or ion beam.

Observed differences in MS signal ratio intensities between both sides of the intact or treated leaves do not take into account these two effects, which cannot be quantified. Despite the additional treatment to remove surface wax by dipping the leaves in chloroform, tropane alkaloid could still be identified on both sides of the leaves but no relative quantification between lower or upper leaf surfaces could be achieved.

Analyzing cross sections appears to be the only method so far to accurately localize tropane alkaloids in *E.coca* leaves, and this has been done by ToF-SIMS imaging.

The interpretation of **hair** analysis (by MSI) is complicated. Beyond some potential instrumental limitations (e.g. spatial resolution), hair analysis suffers from an important intra- and inter-individual variability that cannot be neglected. This variability comes from:

i) instrumental variability, which is of ca. 25% (see discussion Chapter 3, Part 4a., i.e. hair analysis by MALDI-SRM/MSI), ii) biological variability, as 5-20% of hair from the same individual are in different growth phases. These different growth phases do not represent the same time frame within a single individual, leading to some kind of ‘fuzziness’ in time correlation when analyzing hair strands or several single hair samples. In addition, differentiation of the different hair growth phases is not possible with cut hair since the root is missing, which is the case within the framework of forensic investigations (i.e. to keep intact the physical integrity of individuals). In other words, even with the most sophisticated analytical tool that provides ultra-high resolution (e.g. SIMS), one cannot determine accurately when a given drug has been taken due to biological limitations. To illustrate, the sampling may influence the result this way: a 1 mm-shift in the cutting region from one single hair sample to another may shift the time frame by 3-4 days. It results that a better

understanding of hair growth and drug incorporation mechanisms is required. Herein, ToF-SIMS provides some useful information, as specified above; information that can still be investigated further.

As far as I am concerned, hair analysis in general and MSI in particular is a powerful for providing long-term information, to determine how consumption evolves over a given period of time and relative quantification seems to be sufficient in this case. The use of absolute quantification to determine the “true concentration” of drugs at a precise given time point would make no sense, as it could not be correlated to actual time of consumption. In forensics, these surveys are useful for characterizing the behavior of an individual consumer, as a follow up of abstinence from illicit drug use after a crime has been committed. So, the relative quantification of drugs of abuse in hair by MSI for forensic investigations fits for purpose and the MALDI-SRM/MSI platform allows for a screening performed in rapid fashion all along the hair sample.

Beyond sophisticated analytical tools available to respond to a specific biological question, it is clear that relevant answers cannot be made without the knowledge of the biology of the tissue in question.

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List of publications

This thesis is based on following peer-reviewed articles:

“Gas-phase separation of Drugs and Metabolites using Modifier-Assisted Differential Ion Mobility Spectrometry Hyphenated to Liquid Extraction Surface Analysis and Mass Spectrometry”

T. Porta, E. Varesio and G. Hopfgartner, *Anal. Chem.* **2013**, 85 (24), 11771–117793 (**Chapter 4, Part 3**)

“Single hair cocaine consumption monitoring by mass spectrometric imaging”

T. Porta, C. Grivet, T. Kraemer, E. Varesio and G. Hopfgartner, *Anal. Chem.* **2011**, 83, 4266-4272 (**Chapter 3, Part 4a**)

“Alternative CHCA-based matrices for the analysis of low molecular weight compounds by UV-MALDI-tandem mass spectrometry”

T. Porta, C. Grivet, R. Knochenmuss, E. Varesio and G. Hopfgartner, *J. Mass Spectrom.* **2011**, 46, 144–152 (**Chapter 3, Part 2**)

Non peer-reviewed articles:

“Molecular imaging by mass spectrometry: application to forensics”

T. Porta, E. Varesio, T. Kraemer and G. Hopfgartner, *Spectroscopy Europe*, **2011**, 23 (5), 6-12 (**Chapter 2**)

Other non peer-reviewed articles:

“Imagerie par spectrométrie de masse pour la detection de produits stupéfiants”

G. Hopfgartner, T. Porta, L.-A. Leuthold and E. Varesio, *L'Actualité chimique*, Octobre – Novembre **2013**, n°378-379, 94-99

Contributions to conferences and seminars

Oral Presentations

T. Porta, E. Varesio and G. Hopfgartner, *"Gas-phase separation of drugs of abuse and their metabolites from tissue sections by differential ion mobility spectrometry"*, 39th International Symposium on High-Performance-Liquid-Phase Separations and Related Techniques, Amsterdam, The Netherlands, June, **2013**

T. Porta, E. Varesio and G. Hopfgartner, *"Gas-phase separation of Drugs and Metabolites using Modifier-Assisted Differential Ion Mobility Spectrometry after Liquid Extraction Surface Analysis"*, 61th ASMS conference on Mass Spectrometry and Allied topics at the Conventional Center of Minneapolis, MN, USA, June, **2013**

T. Porta, B. Flinders, E. Varesio, Ron M.A. Heeren and G. Hopfgartner, *"Multimodal analysis of cocaine in hair by fast acquisition MALDI-SRM and high resolution ToF-SIMS imaging"*, COST BM1104 – Workshop on "Emerging technologies and multimodal imaging", Vienna University of Technology, Vienna, Austria, February, **2013**

T. Porta, E. Varesio and G. Hopfgartner, *"Assessing the relevance of Surface Sampling based on Liquid Extraction coupled to Differential Ion Mobility for Quantitative Mass Spectrometry Imaging"*, 29^{èmes} Journées Françaises de Spectrométrie de Masse, Orléans, France, September, **2012**

G. Hopfgartner, A. Lesur, T. Porta and E. Varesio, *"What can we learn from MALDI-SRM/MS?"*, 1st Ourense Conference on Imaging Mass Spectrometry, Ourense, Spain, September, **2012**

T. Porta, E. Varesio and G. Hopfgartner, *"Direct QUAL/QUAN analysis of isomers in tissue sections by Liquid Extraction Surface Sampling hyphenated to Differential Ion Mobility – Tandem Mass Spectrometry"*, PhD day of the School of Pharmaceutical Sciences, University of Geneva, Geneva, Switzerland, June, **2012**

T. Porta, T. Kraemer, E. Varesio and G. Hopfgartner, *"MALDI imaging and liquid extraction surface analysis – differential ion mobility spectrometry mass spectrometry: application to forensics"*, 26^{ème} séminaire en Sciences Pharmaceutiques, Analytics on the Top, Zermatt, Switzerland, September, **2011**

T. Porta; E. Varesio, G. Hopfgartner and T. Kraemer, *"MALDI-mass spectrometric imaging – analysis of cocaine and metabolites in a single hair"*, SOFT TIAFT Meeting, San Francisco, September, **2011**

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T. Porta, T. Kraemer, E. Varesio and G. Hopfgartner, *"Fast determination of drugs of abuse in human tissue sections by MALDI mass spectrometric imaging"*, 58th ASMS conference, Salt Lake City, UT, USA, **2010**

T. Porta, C. Grivet, E. Varesio and G. Hopfgartner, *"Alternative CHCA-based matrices for the low molecular weight compounds analysis by UV-MALDI-MS"*, 57th ASMS conference, Philadelphia, PA, USA, **2009**

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“Success is not final, failure is not fatal:
it is the courage to continue that counts”

Winston Churchill (1874 - 1965)