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Potential of MALDI imaging for the toxicological evaluation of environmental pollutants

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Abstract

Risk assessment related to the exposure of humans to chemicals released into the environment is a major concern of our modern societies. In this context, toxicology plays a crucial role to characterize the effects of this exposure on health and identify the targets of these molecules. MALDI imaging mass spectrometry (IMS) is an enabling technology for biodistribution studies of chemicals. Although the majority of published studies are presented in a pharmacological context, the concepts discussed in this review can be applied to the toxicological evaluation of chemicals released into the environment. The major asset of IMS is the simultaneous localization and identification of a parent molecule and its metabolites without labeling and without any prior knowledge. Quantification methods developed in IMS are presented with application to an environmental pollutant. IMS is effective in the localization of chemicals and endogenous species. This opens unique perspectives for the discovery of molecular alterations in metabolites and protein biomarkers that could help for a better understanding of toxicity mechanisms. Distribution studies of agrochemicals in plants by IMS can contribute to a better understanding of their mode of action and to a more effective use of these chemicals, avoiding the current concern of environmental damage.
Introduction

A wide variety of exogenous molecules such as therapeutic drugs, agriculture and industrial chemicals, food additives and cosmetics are present in our environment (food, air, water and soil, animals and plants). The continuous exposure of humans to these chemicals released into the environment raises crucial questions about its impact on health. Different effects on respiratory, neurological immunological and endocrine systems are regularly reviewed (e.g., [1-6]). This recent awareness calls for the development of new analytical techniques that are able to provide rapid information on the tissular and molecular targets of these chemicals and their metabolites and also to study the biological processes implicated in their mechanisms of toxicity.

The risk assessment and the characterization of adverse effects of environmental pollutants on human health is therefore a major issue. Since 1981, the guidelines for the testing of chemicals are published and regularly updated by the Organization of Economic Co-operation and Development (OECD). They gather internationally accepted methods used by government, university and industrial laboratories to determine the safety of chemicals by evaluating their physico-chemical properties, degradation and accumulation in the environment, effects on biotic systems (ecotoxicity) and on human health (toxicity). Toxicity tests must evaluate the different adverse effects (skin/eye irritation, mutagenicity, carcinogenicity, neurotoxicity, developmental toxicity, one- and two-generation reproduction, etc.) observed after oral administration, inhalation or contact (via the skin) of each studied molecule at different dose levels (repeated, chronic or subchronic doses) [7]. As defined by the OECD test guidelines, toxicokinetic studies must be conducted to provide information on the absorption, distribution, metabolisation and excretion (ADME) of the tested molecule.
Toxicokinetic studies are generally based on the use of radiolabeled compounds and on the analysis of tissue homogenates or biological fluids by liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). Quantitative whole-body autoradiography (QWBA) is a commonly used reference technique for distribution studies that involves the administration of the radiolabeled molecule, providing robust, sensitive and quantitative information. However, as QWBA only monitors radioactivity, the parent molecule cannot be differentiated from its metabolites or degradation products in situ. Therefore, metabolites are generally identified from the analysis of tissue homogenates or biological fluids, leading to the loss of information concerning their in situ localization. Since the last decade, IMS [8] has appeared as a powerful alternative for distribution and metabolism studies of small molecules (< 1 kDa) [9-11] and could be also useful for the toxicological assessment of chemicals released into the environment.

In this review, we discuss the advantages of IMS for studying the distribution of small molecules and their subsequent metabolites. The future integration of IMS in toxicity tests requires the development of solid quantitative methods. The different approaches proposed for the quantification of small molecules by IMS as well as an example of its application to an environmental pollutant (chlordecone) are presented. The now well-known potential of IMS for the discovery of protein biomarkers that could help for a better understanding of toxicity mechanisms is also addressed. Although this review is mainly focused on toxicological aspects, the use of IMS for studying the distribution and metabolism of pesticides in plants is also presented. Indeed, this particular application of MALDI imaging is of great interest in the field of environmental research as it can help for a better use of pesticides or for the
development of new molecules. Finally, the limits, perspectives and future applications of IMS to toxicological evaluation of chemicals released into the environment are discussed.

**IMS simultaneously localizes the parent molecule and its metabolites**

IMS offers some unique advantages over QWBA for the analysis of compounds in tissues that are conventionally used during toxicokinetic study of a molecule. This technology allows the localization of the molecule of interest without radioactive labeling, preserving the spatial information that is lost in conventional studies where tissue extracts or biological fluids are analyzed. The major advantage of IMS for distribution studies is its capacity to detect *in situ* the parent molecule and its metabolites without target specific reagents [12]. This represents an important time and cost saving compared to QWBA that necessitates the attachment of radiolabels at different positions of the molecule of interest to get a complete picture on metabolite formation and distribution. IMS is thus complementary to conventional techniques and could become a powerful tool to perform a faster and less expensive screening of the distribution of small molecules and their metabolites in tissues.

The majority of publications report the use of IMS for studying the distribution of therapeutic drugs and their metabolites in whole-body sections or in isolated organs (for review, see [9, 11, 13, 14]). Although these studies are generally presented in a pharmacological context, the methods could be easily transposed to the toxicological evaluation of chemicals released into the environment. In 2006, IMS was successfully used for the first time by Kathib-Shahidi *et al.* [15] to simultaneously study the distribution of an antipsychotic drug (olanzapine) and two metabolites in whole-rat sections at various times after administration (Figure 1). The distributions of olanzapine and its metabolites
determined by IMS were perfectly coherent with previous data obtained by QWBA. In 2007, Stoeckli et al. directly compared QWBA and IMS with experiments carried out on a rat dosed with a radiolabeled compound [12]. The authors concluded that QWBA was more sensitive and more quantitative but concluded that IMS provided the simultaneous localization and unambiguous identification of the parent drug and its metabolites in the same section.

Specificity is a crucial parameter for distribution studies and can be improved in different ways depending on the type of analyzer coupled to the MALDI ionization source. Single reaction monitoring (SRM) available on quadrupoles and ion traps can be used to monitor a fragment ion originating specifically from the precursor ion of interest and constituting a SRM transition [15, 16]. This targeted analysis mode was successfully used by Khatib-Shaidi et al. for the distribution study of olanzapine and two metabolites presented in Figure 1 [15]. Ion mobility can also be efficient in the separation ions according to their gas phase mobility. Trim et al. were thus able to differentiate an anticancer drug (vinblastine) from isobaric lipids with this technology [17]. As a result of their high resolving powers, Fourier transform (FT) mass spectrometers (i.e., Orbitrap and FT-Ion Cyclotron Resonance) are extremely powerful in separating ions of small molecules from interfering matrix clusters or endogenous species and thus gain in specificity [18, 19]. Moreover, the high mass accuracy of this type of instrument allows the measurement of exact masses. The concept of mass defect, defined as the difference between the nominal mass and the exact mass of a molecule, can thus be exploited and constitutes a significant aid for the identification of metabolites of a parent molecule [20]. As an example, the high mass accuracy of a MALDI-LTQ-Orbitrap system was advantageously exploited by Shahidi-Latham et al. to identify an unexpected metabolite of reserpine, an antihypertensive drug, in whole-rat sections using mass defect filters corresponding to common biotransformation analysis. The identity of this
uncommon metabolite was then confirmed by MS/MS fragmentations [21]. The ultra-high mass accuracy and the precision of isotopic profiles typical of FT-ICR mass spectrometers open further possibilities as they can be used to determine the elemental composition of small molecules, thus greatly facilitating their identification. Cornett et al. demonstrated the benefits of FT-ICR mass spectrometers to obtain highly specific MALDI images of olanzapine in rat kidney and liver and of imatinib and its metabolites in mouse glioma [22]. Elemental compositions corresponding to metabolites were also highlighted. The identity of these metabolites was then confirmed by MS/MS fragmentations. In conclusion, combined with a confirmation by MS/MS fragmentations, the high performance levels of FT-ICR mass spectrometers are effective in providing highly specific MALDI images but also in the identification of \textit{in situ} metabolites. The cost of ultra-high resolving power of FT-ICR mass spectrometers is the higher detection time compared to imaging systems based on time-of-flight (TOF) mass analyzers. From our experience, with the same laser shot frequency, the acquisition times are about three times longer with a 7T FT-ICR than with a TOF/TOF analyzer operating in reflectron mode.

\textbf{Quantitative IMS: a prerequisite for its use in toxicological evaluation of chemicals}

\textit{The different methods of quantitative IMS}

The development of IMS for toxicological evaluation of small molecules absolutely requires solid quantification methods. However, such quantification is complicated with IMS for several reasons [23]. First, endogenous species present in a tissue section can affect the intensity of the ion of interest and can lead to varying ionization efficiency across the section. Second, the heterogeneity of matrix crystallization can induce signal variations.
Finally, depending on the physico-chemical properties of the studied molecule and the composition of the matrix solution, the extraction of the compound of interest occurring during matrix application can be more or less efficient. Quantification by IMS is thus very challenging but is also essential for a future integration of IMS in toxicological evaluation. Consequently, the development of quantitative IMS methods has been the subject of very intensive researches over the past years [16]. The majority of published examples concern the quantification of drugs [24-29]. Thus, levels of tiotropium contained in lung of rats after inhalation of this bronchodilator were estimated by correlation with known amounts of tiotropium deposited on a control tissue section. The results were in good agreement with LC-MS/MS quantification performed on tissue extracts [18, 28]. Propranolol (a beta-blocker) and olanzapine were quantified by IMS in whole-body rat sections with a method based on the calculation of a tissue extinction coefficient (TEC) allowing the compensation of ion suppression effects for each organ (TEC) [30]. Koeniger et al. also used olanzapine to demonstrate that the absolute quantity measured by LC-MS in serial liver sections could be correlated with the signal intensity detected by IMS [27]. A similar method was applied to the quantification of raclopride, a dopamine D2 receptor selective antagonist, in several mouse organs [31]. These different approaches without internal standard are well adapted to the quantification of chemicals in homogeneous tissues but results are more variable for highly structured tissues [30, 32].

Variation in ionization efficiency effects across the tissue section and matrix crystallization heterogeneities can be corrected by approaches using an internal standard [32]. With normalization of the signal through use of an internal standard, relative standard deviations of approximately 10-15%, compatible with the OECD guidelines, for the testing of chemicals can be achieved [16, 26, 32, 33]. These approaches differ mainly in the method used for the
deposition of the internal standard that is generally the studied molecule labeled with stable isotopes (D, $^{13}$C, $^{15}$N, etc.). For the quantification of cocaine, Pirman et al. deposited the internal standard onto the ITO slide prior to the fixation of the tissue using a micro-spotter [29]. Kallback et al. quantified imipramine and tiotropium in the brain and lung of dosed rats by introducing the internal standard in the matrix solution deposited on the tissue section using a spraying device [26]. They also developed the msiQuant software available at http://www.maldi-ms.org for the signal normalization and the calculation of calibration curves.

A validated quantitative IMS approach was recently developed in which an isotopically labeled standard was deposited using a robotic spotter in an array of microspots (~200 μm in diameter) across a liver tissue section dosed with a drug [34]. Calibration standards were deposited on a non-dosed tissue section and analyzed in the same way as those on the dosed tissue. After the standards were deposited, matrix was applied to extract the internal standard and the analyte of interest from the dosed tissue section and the calibration standards using the same method. These methods were further validated with mouse kidney following in vivo treatment of the animal with promethazine (PMZ) [35]. Since drugs are filtered out of the blood in the cortex of the kidney, higher concentrations of PMZ were expected to be detected in the renal cortex than the renal medulla. Calibration standards (5-75 μM PMZ with 20 μM $^{2}$H$_{3}$-PMZ) were deposited using a robotic spotter onto a non-dosed tissue section in both the medulla and the cortex. A quality control solution (25 μM PMZ with 20 μM $^{2}$H$_{3}$-PMZ) was also deposited to ensure the method produced less than 15% error. The dosed tissue section was prepared by depositing 20 μM $^{2}$H$_{3}$-PMZ in a 350 × 350 μm array across the section. Matrix solution (DHB) was deposited onto all calibration and internal standard microspots. The microspots were analyzed by IMS in the MS/MS mode to
isolate and fragment the precursor ions for both PMZ and $^2$H$_3$-PMZ in a large isolation window ($m/z$ 286.6 ± 3.0). The quantitative image (Figure 2) obtained shows the distribution of PMZ in which each microspot represents a concentration of PMZ. It can be seen that PMZ is localized to the cortex of the kidney. The quantitative IMS data for the entire tissue section was averaged and compared to HPLC-MS/MS data from extracts of the same kidney in which there was 98.8% agreement.

Application of quantitative IMS to an environmental pollutant (chlordecone)

The methods for quantitative IMS that have been developed for therapeutic drugs can also be applied to environmental pollutants. Lagarrique and coworkers [33] have recently demonstrated that IMS could be used for the in situ absolute quantification of chlordecone in the mouse liver. Chlordecone is an organochlorine pesticide that was extensively used in the French West Indies in banana plantations from 1973 to 1993. Its use has led to a persistent pollution of the environment and to the contamination of the local population for several decades with effects demonstrated on human health [36-38]. Our quantification method combines the normalization by an internal standard added to the matrix solution and the correlation with an orthogonal technique (e.g. GC) to achieve in situ absolute quantification (Figure 3A). Indeed, in the particularly complex case of chlordecone, the quantities determined by IMS were not directly absolute quantities. This was probably due to different conversion rates of chlordecone into its hydrate form (the only form detectable by MALDI MS and/or to different extraction yields of chlordecone between the exposed section and the calibration spots. After establishment of the correlation curve between the data from IMS and GC, absolute quantitation were deduced from IMS data with the
Advantage of preserving in situ localization. This quantitative method allowed us to reveal a particular localization of chlordecone in the pathological liver of a chlordecone and CCl₄ treated mouse (Figure 3B). We were also able to determine the profile of chlordecone accumulation in the mouse liver at varying times of exposure (Figure 3C). The sensitivity of this method was sufficient to detect chlordecone at a dose conventionally used for toxicological studies in rodents. Moreover, as shown in other publications, IMS offers the advantage of being able to extract quantitative information at the pixel level [16].

Investigation of mechanisms of Toxicity

The major goal of most toxicological studies is to understand the mechanisms of action of toxicants on biological systems. This is also evermore important in the case of environmental pollutants whose toxic mechanisms are generally not fully elucidated. Biological functions affected by exposure to a toxicant can be revealed by the identification of proteins and metabolites present in altered expression levels. For proteins, this is addressed using toxicoproteomics that has already been applied to a range of molecules such as drugs, natural products, industrial chemicals, metals and metalloids, nanoparticles and nanofibers (for review, see [39]). This approach is effective but relies generally on the analysis of tissue homogenates or biological fluids that leads to the loss of analyte localization. IMS can conserve this information without the need for complex sample preparation [40].

The potential of IMS for the discovery of potential biomarkers of pathologies has been demonstrated in several articles [41-46] and this approach can be applied for the discovery of toxicity biomarkers that could help for the elucidation of mechanisms of action. Meistermann et al. revealed an accumulation of a fragment of transthyretin (Ser²⁸-Gln¹⁴⁶)
that could be used as a marker of nephrotoxicity in the cortex of rat kidney after
administration of the antibiotic gentamicin [47]. As gentamicin binds to megalin, which is a
ligand for transthyretin, the authors suggest that a preferential binding of megalin with
gentamicin could prevent the efficient reabsorption of transthyretin into the bloodstream.
Karlsson et al. observed a dose-dependent reduction of myelin basic protein in the stratum
of adult rats brain neonatally exposed to the cyanobacterial toxin β-N-methylamino-L-
alanine (BMAA) [48]. This result, revealed by IMS and confirmed by immunohistochemistry,
indicates that the neonatal exposure to BMAA could reduce or modify the myelination of
axons in the striatum. This could explain cognitive impairments observed after exposure to
BMAA in previous studies. Finally, Bauer et al. identified α-defensins (DEFAs) as protein
markers of response to treatment of breast cancer patients with neoadjuvant paclitaxel and
radiation using histology-guided MALDI profiling [49, 50]. After treatment with paclitaxel and
radiation, DEFAs were found to be overexpressed in tumors of patients with pathologic
complete response compared to patients with residual disease. This approach can be applied
to the discovery of proteins as markers of the toxicity of environmental pollutants. A careful
study of biological functions and molecular processes associated to these proteins could
contribute to a better understanding of toxicity mechanisms.

Understanding the mode of action of agrochemicals in plants

Another dynamic field for the application of IMS directly related to environmental questions
is the use of this technology to study the absorption, the distribution and the metabolism of
agrochemicals in plants. This particular application is vital for a better understanding and
better control of the action of agrochemicals. However, it is important to note that the
presence of cuticles and plant cell walls at the plant surface can make IMS of plants very
difficult [51, 52]. They obtained molecular ion images of the fungicide azoxystrobin on the
surface of soya leaves and in the stem of soya plants at different times after fungicide
application. This study demonstrates the feasibility of IMS of pesticides in plants and
revealed the absorption of azoxystrobin from the roots toward the stem of soya plants.
Annangudi et al. used IMS to study the distribution of three fungicide residues
(epoxiconazole, azoxystrobin, and pyraclostrobin) on the surface of wheat leaves [53]. In this
study, the three fungicides were applied on leaf surface using a track sprayer system in order
to simulate field application conditions. Their results characterized the behavior of
agrochemicals on leaf surface after pesticide application. In 2009, Anderson et al. optimized
the protocol of sample preparation to determine the distribution of nicosulfuron, a
sulfonylurea herbicide extensively used in the cultivation of cereals, in sunflower sections
taken from different regions of the stem after root or foliar uptake (Figure 4) [54]. This study
revealed different localizations of nicosulfuron and a phase 1 metabolite depending on the
uptake type. This work also studied the distribution of four other sulfonylurea herbicides
(chlorimuron-ethyl, chlorsulfuron, imazosulfuron and pyrazosulfuron-ethyl) in sunflower
after foliar uptake [55]. Depending on the herbicide, the amplitude of the translocation from
the application point towards the growing tips and/or towards the roots was shown to be
very different.

Limitations and future perspectives

IMS has a great potential for application to studies involving the distribution and the
metabolisation of chemicals released into the environment but also to better elucidate their
mechanisms of action. Nevertheless, technological developments are still needed for further integration of IMS into this research field. First, the sensitivity of IMS may not be sufficient to detect the studied molecule even in some cases where high doses of toxicant are used to induce observable effects. This can be due to the intrinsic ionization yield of the molecule of interest and/or to ion suppression effects and poor ion fragmentation for MS/MS studies. Continuous accumulation of selected ions (CASI) available on some FT-ICR instruments can be used to improve sensitivity and dynamic range. This analysis mode, which consists in the selective accumulation of ions in a small mass range around the ion of interest before ion introduction in the ICR cell, was used by Groseclose et al. to significantly improve the detection sensitivity of lapatinib and nevirapine in rat liver [24]. Depending on the molecules, the limit of detection can be enhanced by a factor 10-50 with CASI [56].

The development of IMS for distribution studies performed on whole-body sections benefit from the reduction of acquisition times. New instruments have achieved laser shot frequencies of up to 10 kilohertz, enabling an enhancement of the lateral resolution of tissues because many more smaller pixels can be acquired in a short period of time. Images have been reported to be acquired with lateral resolutions of 20 μm [57] and 5 μm [58], important advances for the identification of cellular targets in the context of toxicological evaluation of chemicals released into the environment.

Although several methods have been developed for the identification of proteins detected by IMS, it remains tedious. Reported approaches are based on top-down [41, 57, 59] or bottom-up [43, 60] analysis of tissue homogenates or directly from tissue sections using on-tissue enzymatic digestion [61] or in-source decay [62]. Nevertheless, unraveling the molecular complexity of tissue samples remains a daunting task. The use of high-resolution
mass spectrometers [63] or coupling MS with ion mobility [64] holds great promise in helping overcome this issue.

**Conclusion**

The major application of IMS to address environmental questions is the analysis of the distribution studies of chemicals released into the environment and their metabolites in animals or in plants and the discovery of toxicity biomarkers to better elucidate mechanisms of action. The direct application of IMS to chemicals released into the environment is just emerging and will undoubtedly expand in the coming years. Indeed, since technological and methodological developments are still underway, different concepts such as distribution studies and biomarker discovery initially developed mainly for therapeutic drugs are now sufficiently advanced to be applied to chemicals released into the environment.

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Figures

**Figure 1:** Simultaneous localization of olanzapine and its metabolites at 2 h post-dose in a whole-rat sagittal tissue section by IMS. A) Optical image of the dosed rat tissue section with organs outlined in red, and MALDI images of B) olanzapine (SRM transition m/z 313 → 256), C) N-desmethyl metabolite (SRM transition m/z 299 → 256) and D) of 2-hydroxymethyl metabolite (SRM transition m/z 329 → 272). Adapted from Khatib-Sahidi et al. [15] with permission. Copyright 2006 American Chemical Society.
Figure 2. Quantitative IMS of kidney tissue dosed with PMZ along with an H&E stained serial section for histological comparison. The results from IMS and HPLC-MS/MS were in agreement for the entire tissue section, and the image provides additional information about the localization of PMZ to the cortex region of the kidney. Adapted from Chumbley et al. [34] with permission. Copyright 2016 American Chemical Society.
Figure 3: In situ absolute quantification of chlordecone in the mouse liver by IMS. A) The quantification method was calibrated with different amounts of chlordecone manually spotted on a control section and normalized with an internal standard ($^{13}$C$_{10}$-chlordecone) added in the matrix solution. B) In situ absolute quantification of chlordecone in the pathological liver of a chlordecone and CCl$_4$ treated mouse with the microscopic image of a serial section stained with H&E and the MALDI image corresponding to chlordecone hydrate: the local absolute quantities measured in each necrotic areas are lower than that measured in the healthy tissue (381 $\mu$g/g). C) Accumulation profile of chlordecone in the mouse liver after an exposure of 1, 2, 5, 10, or 25 days at 5 mg/kg bw. Adapted from Lagarrigue et al. [33] with permission. Copyright 2014 American Chemical Society.
Figure 4: MALDI imaging of azoxystrobin on wheat leaf after track sprayer application. Adapted from Annangudi et al. [53] with permission. Copyright 2015 American Chemical Society.
References


Graphical abstract
Highlights

- MALDI imaging mass spectrometry is an enabling technology for toxicological evaluation of chemicals.
- The major asset of IMS is the simultaneous localization and identification of a parent molecule and its metabolites without labeling and without any prior knowledge.
- IMS opens unique perspectives for the discovery of molecular alterations in metabolites and protein biomarkers that could help for a better understanding of toxicity mechanisms.