A review of complementary separation methods and matrix assisted laser desorption ionization-mass spectrometry imaging: Lowering sample complexity☆

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A B S T R A C T

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI) brings unique combined information on molecular identity and molecular distribution of a sample surface. During the past decade, it has matured and is now routinely employed for biomedical tissue sections analysis. However, owing to the high molecular complexity of tissue, MALDI-MSI suffers from ionization suppression effects. This directly results in a reduced ability to detect low-abundant molecular species. At the same time, the spatial resolution of separation techniques can be insufficient for an unambiguous determination of the local composition of a mixture. As analytical separation techniques can significantly reduce ion suppression, and MALDI-MSI has an ability to improve their spatial resolution, the two analytical approaches can successfully be combined in a pursuit of comprehensive local sample composition information. In the following review we summarize strategies of mutually beneficial combinations of MALDI-MSI and different separation techniques and discuss limitations and future developments.

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1. Introduction

Matrix-assisted laser desorption ionization (MALDI) introduced in Karas et al. [1] pushed the borders of laser desorption ionization (LDI) techniques forward by enabling ionization of large (bio)polymers. Ultimately the ability to analyze intact macromolecules led to the emergence of new analytical disciplines such as proteomics and metabolomics. The groundbreaking improvement was based on the use of matrix. Typically a small organic compound, the matrix has a triple impact on the desorption-ionization process. (i) It separates individual molecules of the analyte from each other, thus minimizing analyte aggregation. (ii) Matrix molecules absorb the initial energy of a laser pulse. As a consequence, the molecules of analyte do not undergo direct photon induced fragmentation and organic compounds up to several tens to hundreds of kilo Daltons can be analyzed. (iii) It promotes ion-molecule reactions in the gas phase (MALDI plume), that results in formation of pseudomolecular ions from analyte molecules [2–4].

The most common sample preparation for a MALDI experiment is the dry droplet method: a solution of analyte(s) is mixed with a matrix solution and small droplets of the mixture are spotted onto a metal target. Upon the droplet drying, co-crystals of matrix and analyte are formed. The actual desorption-ionization process is initiated by exposure to one or more laser pulses. Nowadays UV lasers such as nitrogen (337 nm) or Nd:YAG (355 nm) are mostly employed, and also IR-MALDI with lasers emitting at 2.94 μm are used in analytical practice [5]. The laser shots cause desorption of the matrix-analyte cluster ions as well as matrix and analyte neutrals from the laser interaction region. The analyte ions emerge after ion-molecule reactions with the matrix ions (ideally after gas-phase protonation) or cluster desolvation in the MALDI plume [6]. For its pulsed character, MALDI is preferably coupled to time-of-flight (TOF) mass analyzers.

On one hand, the employment of matrix enabled analysis of high molecular mass species, on the other, matrix presence hampers analysis of the compounds at the low end of the mass range. Because matrix is typically a compound with the molecular mass below 500 Da (e.g. sinapinic acid, 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid (CHCA), etc.) the analysis of low molecular mass species (<500 Da) is complicated by the overlapping matrix peaks, that cannot be resolved by TOF-MS. Moreover, matrix molecules tend to form clusters with molecules well up to 1000 Da, thus shifting the overlap problem even further within the mass range. On top of that, the usual MALDI-TOF spectra contain a significant background signal, which is commonly referred to as "chemical noise". This background signal includes both, matrix ions and unresolved ions that span the whole mass range, and further complicate the resulting spectra [3]. Another pitfall of MALDI is the shot-to-shot signal variability which is caused by the heterogeneity of sample spots [7]. Nevertheless, MALDI possesses a couple of auspicious advantages: next to the possibility to analyze large biomolecules, MALDI offers a unique opportunity to scrutinize the spatial organization of various sample probes in a planar layout. As a result, MALDI application fields reach far beyond analyses of deposited droplet mixture.

In 1997, Caprioli and Farmer [8] demonstrated the use of MALDI mass spectrometry (MS) for the exploration of the molecular composition of tissue sections. The term MALDI mass spectrometry imaging (MSI) has officially been used since that time, although its basic principle was already known and applied for MS analysis of, e.g. gels and TLC plates. MALDI-MSI was a logical junction between the MALDI technique that emerged in the late 1980s and secondary ion mass spectrometry (SIMS) imaging that was already well established at the time [9]. During a MALDI-MSI experiment a sample surface is probed with a laser beam in a predefined raster form and a mass spectrum is recorded at each position. As a result, position correlated mass spectra are collected. The final image is defined by the *x*,*y* coordinates within a Cartesian grid, and the intensities of a particular *m*/*z* visualized in a different colour range [10–12]. An image can be constructed for every single *m*/*z* present in the spectra. Common samples analyzed with MALDI-MSI are thin (10–20 μm) tissue sections [13], but in principle any sample thin and flat enough can be explored by MALDI-MSI.

In general, MSI offers several advantages over the other imaging technologies such as positron emission tomography, computer assisted X-ray tomography, nuclear magnetic resonance imaging, quantitative whole body autoradiography (QWBA), or immunohistochemistry (IHC). First of all, MSI does not require any knowledge on the targeted analytes and has no need for any labels. It is a true discovery tool. Furthermore, it allows for highly precise and specific identification of many hundreds of compounds next to each other while preserving their spatial distribution [12,13]. Indeed, the spatial information is one of the most significant contributions of MSI. Nevertheless, MALDI-MSI is affected by the general weak points of MALDI described above, thus suffering from several limitations.

The simplest MALDI-MSI sample preparation protocol is based on cutting a sample (e.g. a mouse brain) into thin sections and covering them with a layer of matrix [13]. The overlap problem of matrix and its cluster peaks is manifested for the molecules within the matching mass range. Moreover, as the laser beam rasters over the sample, all molecules present on the sample surface can theoretically be desorbed and ionized. Some molecules tend to ionize more easily, especially when they are present in a higher amount, hence they contribute to ionization suppression effects. These can, together with a limited dynamic range of MALDI-TOF mass spectrometers, lead to incomplete data since the low-abundant molecular species remain "hidden". Lowering sample complexity before MALDI-MSI can significantly help to minimize the ionization suppression and reveal molecules which remain undiscovered.

Separations represent possibly the most effective way of a reduction of sample complexity. The main merit is the reduction of ionization suppression contributing to improved ionization efficiency of low-abundant analytes [14]. Apart from understanding separation as a simple clean-up process, during which undesired compounds are washed off the sample surface, the principle of separation seems to be inappropriate for MSI purposes. It would require sample homogenization, thus resulting in a loss of spatial information: the essential parameter studied by MSI [15]. However, separation methods can provide valuable complementary information through screening the composition of a portion of the *to-be-imaged* sample and give direction on the further experimental design [16]. From another perspective, the separation resolution of some of the chromatographic and electrophoretic methods such as high performance liquid chromatography (HPLC), thin layer

chromatography (TLC) or gel electrophoresis (GE) can further be improved by the employment of MALDI-MSI. The technique can provide deeper insight into the composition of mixtures by imaging the captured eluent or separated analyte bands. Clearly, a combination of MSI and separation methodologies enables an analyst to obtain comprehensive information on sample composition. In this review we summarize the possibilities for the reduction of sample complexity that improve MALDI-MSI analysis with a particular attention paid to separation methods. In addition, we review various applications for MSI of separated analytes. The described methodologies contribute to our improved understanding of what can and cannot be visualized by MALDI-MSI.

2. Separations for effective sample clean-up

One of the advantages of MALDI-TOF is the possibility to analyze very complex mixtures that are not required to be of high purity, since MALDI is relatively tolerant to the presence of salts and buffers [17–20]. However, these contaminants can contribute to altered crystallization and/or ionization suppression, and reduce signal-to-noise [21,22]. For instance, proteins are often treated with surfactants and denaturant agents that are better desorbed from the sample surface during MALDI analysis than the proteins themselves [22].

Various methods of sample purification preceding MALDI target deposition based on off-line separation, such as dialysis, selective extraction (solid phase extraction, liquid–liquid extraction, etc.), or chromatographic approaches, have been described so far [17,23]. Some of them are further discussed in this review. These conventional methods can, however, be too time-consuming for a quick sample composition screening. Furthermore, they can lead to undesired sample losses.

Membrane based sample supports are used as a fast, effective and straightforward sample purification method, which simultaneously allow for easy sample introduction into a MALDI-TOF mass spectrometer. This approach enables application of a series of washing solutions over the sample spots deposited on top of the membrane. As a result, impurities and contaminants are separated from the molecular species of interest. The principle is also employed for tissue section samples to lower their molecular complexity prior to MALDI-MSI.

2.1. Sample supports for MALDI-MS

A sample support, typically a polymeric membrane, allows for anchoring samples on the support's surface. The analytes (typically proteins or peptides) bind onto the membrane surface, while the impurities can be washed-off by using a series of rinsing solutions applied on top of the sample spots. Also, further digestion or derivatization of the sample in situ, i.e. directly on the membrane surface, is enabled [22–24]. In the final stage, a matrix solution is applied on top of the processed spots and the analyte-matrix co-crystals are left to form. In any stage of the procedure the membrane is typically placed on a standard MALDI target or a metal disc using a thin layer of adhesive or a double sided adhesive tape [23].

2.1.1. Polymeric membranes

An ideal sample support membrane has to (i) allow for binding the analyte upon the membrane surface, (ii) provide sufficient retentive ability during the sample treatment, (iii) enable analytes to resolubilize upon matrix application, (iv) should not add additional complexity to the spectra generated from the membrane, and finally (v) enable the formation of matrix-analyte co-crystals [24]. Various membrane types have been described in the literature so far: nylon [24] and modified nylon (Zetabind™) [24], polyethylene (PE) [21,22], polypropylene (PP) [22] C8 and C18 discs [22], nitrocellulose [24,25], regenerated cellulose [26], polyvinylidene fluoride (PVDF) [17,21,24], and polyurethane [17,23]. The membranes offer different capabilities to bind various samples in diverse amounts and also differ in their resistance to chemicals [24].

The separation of the impurities and contaminants is only one of the advantages which the sample support membranes offer. McComb et al. [17,23] also demonstrated an accelerated digestion of proteins while using a polyurethane membrane for sample deposition. They explain this phenomenon by protein denaturation upon the membrane adsorption. Worrall and co-workers [22] showed the proteins desorbed from PE and PP membranes require lower laser intensity (10% decrease), which results in lower energy ions and better mass resolution. The group also reported on reduced shot-to-shot variability employing these types of membranes. Use of the sample supports for proteinaceous samples is usually well applicable to the analysis of peptides and proteins up to 20 kDa [17,21,26]. When higher molecular mass proteins are analyzed the spectra show either comparable results with those obtained from stainless steel probes [17] or present poorer results [26]. An improvement in this field was reported by Blackledge and Alexander [21], who described suitability of PE membranes for the analysis of high molecular mass proteins (over 150,000 Da). The authors explained the improvement with regard to the morphological features of PE membranes. They emphasize their smaller pores, thus large and moreover relatively flat surface. As a consequence proteins are more uniformly deposited onto the membrane surface without deep penetration into its pores. In the end, spectra of higher quality with better peak shape are generated and sensitivity for high molecular mass proteins is significantly improved.

Next to the list of benefits provided by sample supports, a couple of drawbacks have to be pointed out. For instance, several authors reported on difficulties with too little sample adsorbing upon the membrane surface when applying higher volumes of the samples (1 μL) [23,26]. Washing of the membranes with methanol was recommended, as the solvent might possibly lower the intermolecular forces of the polymeric material, thus allowing the analyte a better contact with the broadened membrane pores [17,23]. Charging of the membrane surfaces appears in MALDI-TOF when non-conductive surfaces, such as polymeric membranes, are employed. The charge that builds up during the ionization process gradually alters the surface potential and hence the final kinetic energy the ions obtain. The ions generated from the insulating membrane surface exhibit changing flight times, which result in loss of signal, resolution and/or accuracy [24,27,28].

2.1.2. MALDI sample supports with a modified surface

Modification of the membrane surface prior to sample deposition can be advantageous in several ways; higher sensitivity, increased spot homogeneity and higher reproducibility. A thin conductive surface modification can be employed for the elimination of sample charging. Such modifications result in improved mass resolution and accuracy as described in the literature [26,27,29–32]. For instance, sample spot size was tackled in two independent experiments based on modification of the probes' surface: Miliotis and colleagues [29,30] applied a layer of a matrix-nitrocellulose mixture on top of standard MALDI targets. Schuerenberg et al. [31] described a probe covered with a hydrophobic Teflon layer and deposited hydrophilic gold anchors. In both cases, sample spot diameter was reduced resulting in more concentrated sample spots, hence in increased sensitivity, whereas improved droplets' homogeneity minimized the shot-to-shot variability. Scherl et al. [27] applied a thin layer of gold on top of a membrane in order to ameliorate the charging phenomenon. This resulted in charge dissipation during MALDI-TOF analysis, thus yielding spectra with higher mass resolution.

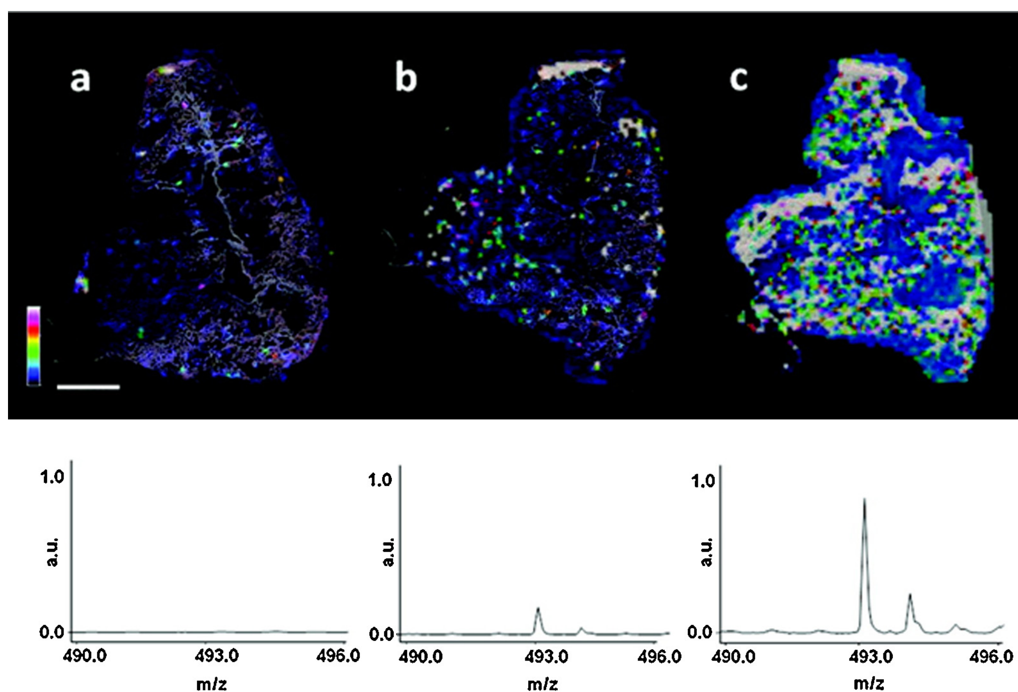

Fig. 1. Matrix assisted laser desorption ionization-mass spectrometry imaging demonstrates the effect of washing lung tissue sections with a buffered solution. Compound X (*m*/*z* 493.2) was administered to rats by in vivo inhalation. Tissue sections were washed by an aqueous solution with adjusted pH. This ensured insolubility of Compound X which therefore remained in the tissue after application of the washing protocol. On the contrary, the soluble endogenous compounds causing ionization suppression were washed away (a) Non-washed tissue section; tissue section washed (b) in 10 mM ammonium acetate at pH 6 and (c) in 100 mM ammonium acetate solution at pH 6. Corresponding summed spectra of the whole tissue sections are shown below the images. (Data displayed in rainbow scale over the same range, scale bar 2 mm.). Figure reprinted with permission from reference [34]. Copyright 2012 American Chemical Society.

Zhang and Caprioli [26] used polymeric membranes precoated with matrix: Regenerated cellulose strips were covered with CHCA and further used as a standard sample support. An advantage of matrix precoated membranes is the elimination of sample dilution which leads to decreased sensitivity. The use of matrix precoated MALDI targets for MALDI-MSI has also been described. To point out, MALDI-MSI suffers from matrix heterogeneity to a particular extent since a homogeneous matrix layer is essential for an accurate MALDI-MSI experiment. A plethora of methods for matrix application, based on spraying, nebulizing or sublimation have been introduced in the pursuit of the "perfect" matrix layer. Yang and Caprioli recently demonstrated the use of precoated sample targets for MSI of lipids [32]. Matrix was applied on the surface of ITO (indium tin oxide) slides using sublimation. Tissue sections were subsequently placed on top of the modified slides and imaged with MALDI-MS. High robustness and reproducibility was reported by the authors.

2.2. Washing protocols for MALDI-MSI of tissue samples

The sample spots on top of a support membrane have several characteristics in common with a tissue section placed on a glass slide. Among them the need for soft washing methods is emphasized. Washing of a tissue section prior to MALDI-MSI is incorporated to separate and selectively remove salts and other undesired molecules from the compounds of interest. The slide with an attached tissue section is either immersed into a beaker with the washing solution, or the solution is applied on top of the tissue using a pipette [13].

Different washing protocols can be applied and are often optimized for the class of analytes studied, so to achieve the most selective separation of the undesired molecules while keeping the molecules of interest in situ. A typical washing protocol for MALDI-MSI includes a series of ethanolic solutions (70–100%), although organic solvents such as chloroform are employed as well. It is always necessary to keep in mind the solubility of the targeted analytes in the solvents used before the washing steps are performed. Using an improper solvent can either cause a delocalization of the analytes, or these can be washed-off the tissue surface. For instance, when lipids are the subject of a tissue study, lipofilic solvents such as ethanol or chloroform have to be avoided. When analyzing small molecules such as pharmaceuticals, washing is not recommended at all [33]. However, a paper on controlled pH washing of tissue sections for an increased limit of detection for small compounds has been published recently [34]. Shariatgorji et al. [34] used aqueous buffered solution with pH adjusted to minimize the solubility of the compounds analyzed. As shown in Fig. 1, the soluble endogenous compounds causing ionization suppression were removed, while the analytes remained within the tissue sample. The authors report minor to no spatial delocalization of the target compounds. Angel et al. [35] also reported on the benefit of a controlled pH washing protocol for more sensitive detection of lipids from brain tissue. It has to be emphasized that the washing procedure has an ability to change the sample properties vastly and this fact should always be kept in mind [33]. For instance, organic solvents can cause significant damage to delicate and fragile tissue [36].

3. Separations within defined spatial dimensions

3.1. Planar layout: one- and two-dimensional separations

MSI is not limited to the analysis of tissue sections [16]; its surface sensitive nature makes the technique suitable for analysis of any probe in a planar layout. MALDI-MSI was for instance used to describe the heterogeneous distribution of analyte molecules within MALDI sample spots [7,37,38]. Weidner et al. [37] and Weidner and Falkenhagen [38] showed heterogeneous distribution of polymers within droplets deposited on a MALDI target by imaging

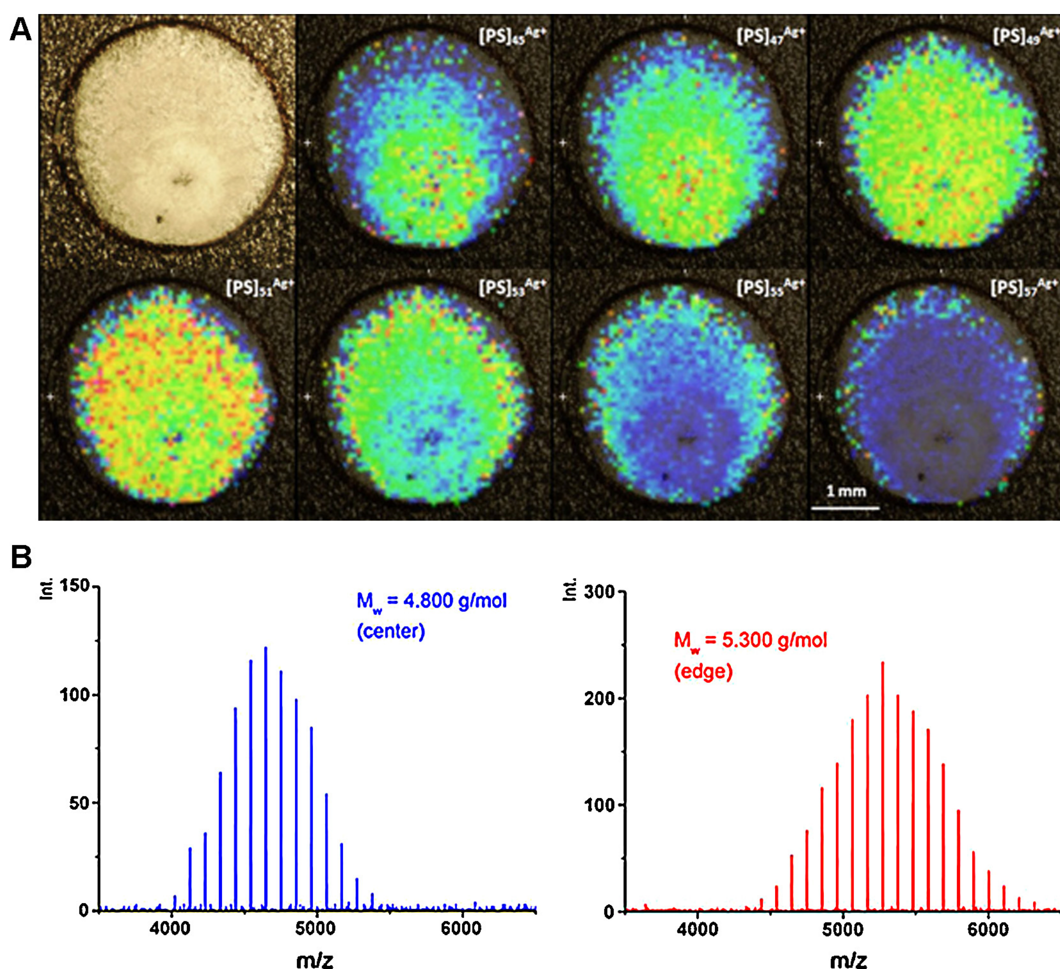

Fig. 2. Matrix assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) demonstrates heterogeneous distribution of polystyrene (PS) homologues within sample spots prepared by dried droplet method. 10 mg/mL of alpha-retinoic acid dissolved in N,N-dimethylacetamid was used as matrix. PS samples were dissolved in the same solvent at concentration of 1.5 mg/mL. Silver-trifluoroacetate was added to enhance PS ionization. (A) Optical image of the dry sample spot is shown on the top left panel. Ion intensity distribution of every second PS homolog from $[PS]45^{Ag+}$ to $[PS]57^{Ag+}$. Lower mass homologues were found with the highest ion intensities in the middle of the dried spot, whereas higher mass species were located on the droplet rim. (B) MALDI spectra collected on the rim and in the centre of the spot showed 13% molecular mass deviation. Figures adapted with permission from reference [37]. Copyright 2011 Springer-Verlag.

the spots with MS. Fig. 2 demonstrates uneven distribution of particular polystyrene homologues within the spots and differences between spectra acquired from the rim and from within the centre of the dry droplets.

Another example of diversity of MSI applications is represented by gels generated with GE and by TLC plates. These techniques are commonly used for the separation of complex mixtures of proteins and various classes of lipids as well as other lower molecular mass compounds, respectively. Deposition of such intricate mixtures on a standard MALDI target would provide an analyst with very complex information and is likely to suffer from ionization suppression effects and low dynamic range. Using membrane sample supports for fast separation of impurities or employing tissue washing protocols does not necessarily lead to a sufficient decrease of sample complexity, and only limited improvement is offered. The impurities are washed-off, but the individual molecular species of lipids and/or proteins remain unseparated. More detailed information on the composition of mixtures can be obtained when separation techniques are employed. Fig. 3 shows a diagram describing briefly how MSI can be involved from two different perspectives:

(1) Direct MALDI-MSI of developed gels and TLC plates can be employed. In such case, MALDI-MSI increases the separation resolution of the methods, thus helping to further lower the complexity of the separated mixtures, and provides additional information on samples composition. Moreover, MSI contributes to increased throughput of the analysis.
(2) The composition of a tissue sample can be addressed into more detail by creating a homogenate and/or extract from an adjacent tissue section. The obtained lipids and/or proteins can further be separated either by GE or by TLC. Once identified, the separated molecular species can be targeted during MSI of a tissue section.

3.1.1. *Gel electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) is a one-dimensional (1D) separation technique used for separation of proteins based on their molecular mass. The proteins are first denaturated by sodium dodecyl sulphate (SDS), which results in a charge state proportional to their molecular mass. The proteins are subsequently separated within the gel in the presence of electric field and their molecular mass determined by the migration distance [20]. Isoelectric focusing (IEF) separates proteins according to their isoelectric point, which is determined by their amino acid sequence. IEF employs an in-gel immobilized pH gradient and electric field. The charge of the protein changes as it migrates through the pH gradient and

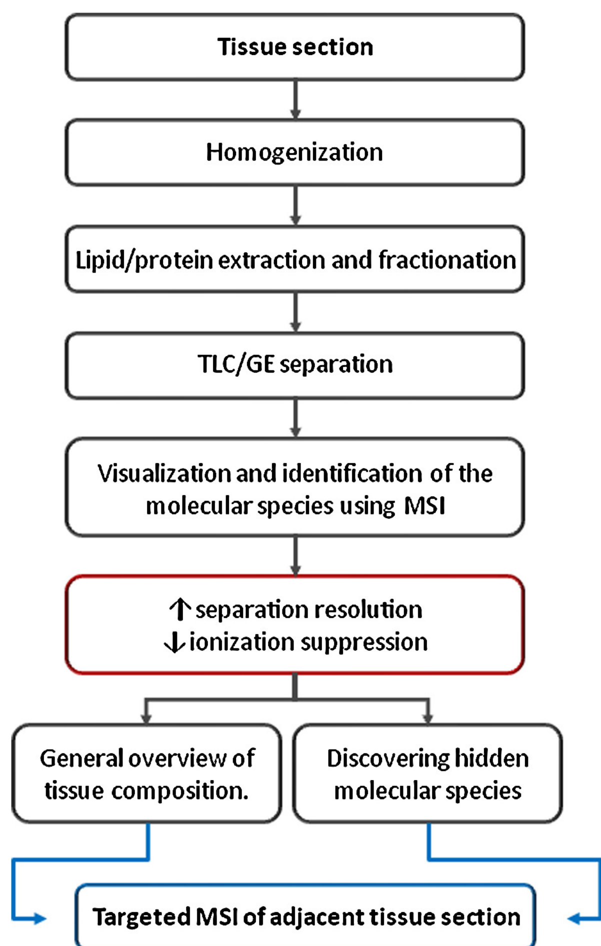

Fig. 3. Workflow for combining matrix assisted laser desorption ionization-mass spectrometry imaging and separation methods to obtain comprehensive complementary information on sample composition.

the migration stops when the protein reaches the pH in which it becomes neutral (isoelectric point) [20]. Two dimensional gel electrophoresis (2D-GE) combines PAGE and IEF to separate proteins within one gel, first according to their isolelectric points and subsequently according to their molecular masses [24].

Following separation, proteins are identified using complementary methods. The methods most commonly used include: use of dyes (such as silver stain, Coomasie Brilliant Blue, amidoblack or Poinceau Red) for proteins visualization and subsequent 2D-GE image database searching; immunostaining; sequencing by Edman degradation; and MS [20,39]. Patterson and Aebersold [28] reported in detail on the mass spectrometric identification of proteins from 2D-GE gels.

MS is a powerful tool for protein identification, and can alleviate most of the limitations of the other methods, such as low sensitivity or potential unavailability of antibodies. For subsequent MALDI-MS analysis, the individual protein bands can either be excised or eluted from the gel and deposited on a MALDI target, or the whole gel can be blotted onto a support membrane, thus obtaining a positive imprint of the separated protein bands. (The latter approach is further discussed in Section 3.2.2 of this review as a part of the three-dimensional separations.) However, to improve the speed of analysis, elimination of the excision and blotting steps is desired [40], and call for direct MALDI-MS(I) of the gels is emphasized [41]. For instance, Ogorzalek Loo et al. [41] described MALDI-MS approach for characterization of proteins directly from polyacrylamide gels. They claim that their approach is suitable for both 1D and 2D gel separations. A requirement of significant importance, which cannot be taken for granted, is that the gel thickness does not exceed 10 μm. The authors point out and discuss in detail the fragility of thin gels. As reported, increase of mechanical stability can be achieved by soaking the still wet unstained gels into sinapinic acid solution. Sinapinic acid is believed to act as a plasticizer.

Even though some authors proclaim 2D-GE being the method with the highest resolution for protein separation [20], the actual separation power is often insufficient, thus resulting in single spots containing more than one protein species [42]; more than 10% of the protein bands actually contain more than one protein [40]. The group of Yang et al. [42] resolved this problem by subjecting the spots excised from 2D-GE gel to complementary liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Contribution of MSI to direct analysis of the 2D-GE gels would help to improve separation resolution by revealing spots of co-migrating proteins or identical proteins which migrate to different positions within the gel [42]. Gels after protein separation by IEF were rastered with a laser beam (step size of 0.3 mm corresponded to roughly 500 mass spectra from a single band [40]). Subsequently a dedicated software was employed to obtain so called “virtual 2D gels” [40,43]. The generated 2D images were based on isoelectric point and molecular mass determined by MALDI-MS instead of SDS PAGE [41,43]. Walker and co-workers [40] compared this approach with the conventional 2D-GE and showed that the virtual 2D gels enable identification of proteins, which remain unresolved under 2D-GE conditions. They further report the accuracy of molecular mass determination is 100–500 times better for the virtual 2D gels [20,40]. An example of an acquired virtual 2D gel is shown in Fig. 4.

It has to be pointed out that successful MALDI-MSI analysis of gels requires a lot of practical considerations. Particular attention has to be paid for instance to vacuum compatibility and stability of the gel, to washing-off the crystallization disrupting contaminants while preserving the distribution of the proteins within the gel, or to a proper matrix-analyte co-crystals formation [40]. Charging phenomena have been observed, since gels act as insulators [44]. These factors also limit the successful application of MALDI-MSI for the analysis of 2D-GE gels.

3.1.2. Thin layer chromatography

TLC is based on distribution of an analyte between two phases: a stationary phase, anchored on a solid support, and a mobile phase transported by capillary forces through the layer of the stationary phase [45]. 2D separations represent an attractive application of TLC. In such case, two systems of mobile phase are used in a subsequent order to develop a single TLC plate [46], thus increasing the peak capacity and separation resolution.

The scope of the most routine TLC applications includes fast monitoring of drugs of abuse and toxic substances in biological fluids, water purity surveys, quality control in pharmaceutical industry [45], and a rapid screening of impurities of the in-house synthesized compounds. It is also widely used in lipid research for separation of individual lipid classes, see also Fig. 5 [47]. The scope of the analytes typically targeted by TLC is represented by molecular species with mass roughly below 1000 Da [44]. The traditional detection methods for TLC are based on UV and fluorescence detection or on the use of colour reagents and derivatizing reactions. Another group of detection systems for TLC is based on optics, e.g. scanning densitometry or video densitometry, the latter one replaced the antiquated technique of photographing the TLC plates [45]. MS(I) falls to the third category of TLC detection systems.

Since development of electrospray ionization (ESI), coupling HPLC to MS has become well established and routinely used. Also

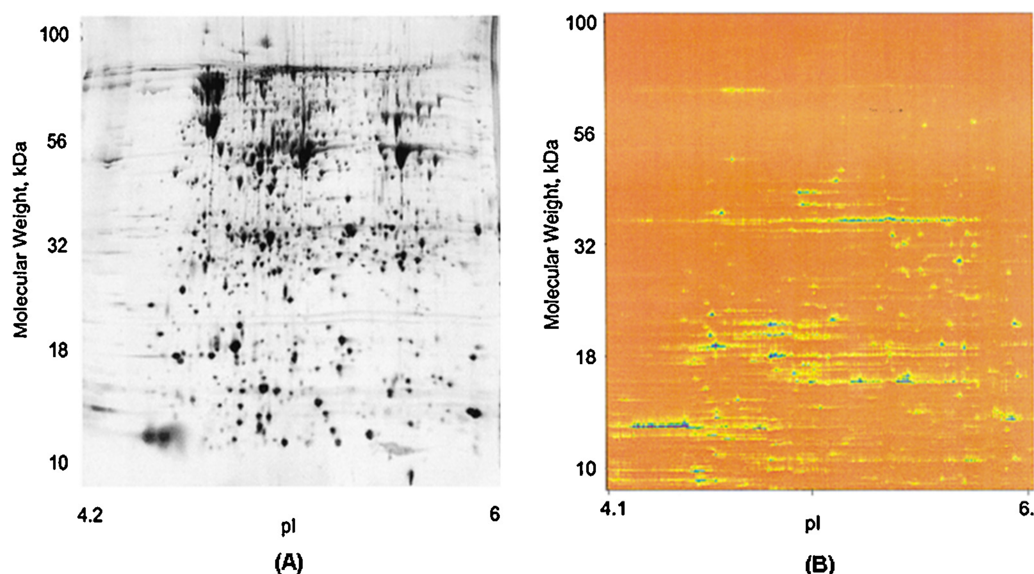

Fig. 4. Comparison of two visualization techniques for separated *E. coli* whole cell extracts. (A) Gel after a conventional two-dimensional gel electrophoresis (2D-GE), proteins visualized with silver-staining and (B) virtual 2D gel acquired by MS scanning over in-gel protein bands separated by isoelectric focusing. Specialized software tool was used to reconstruct the 2D image. Figure adapted with permission from reference [40]. Copyright 2001 WILEY-VCH.

TLC can be coupled to ESI-MS: The off-line TLC-ESI mode is based on solvent elution or extraction from the TLC separated bands and a subsequent injection of the independent fractions into a mass spectrometer [48]. Obviously, such an approach is very laborious and time consuming [45,48]. Moreover, the extraction/evaporation procedure can lead to significant sample losses and a sensitivity decrease. On the contrary, the on-line TLC-ESI interface ensures faster, more robust and sensitive detection. However, the price for reduced risk of sample losses is the requirement for highly sophisticated instrumentation [46]. For these reasons, the most efficient way for direct TLC-MS coupling is based on desorption ionization techniques such as MALDI [46,48] (or DESI). With MALDI the analytes can directly be desorbed from the plate surface omitting the imprecise and lengthy evaporation or elution process.

In general, the compatibility of TLC plates for direct MALDI-MS analysis is higher than that of gels. The pitfalls of the interface are mainly represented by charging phenomena [44], and possible contamination of the mass spectrometer by silica residues [49]. Unlike in GE, use of matrix represents a major limitation in MALDI-TLC with regard to the molecular masses of the analytes typically studied. Application of the solvent based matrices also causes band spreading. Moreover, attention has to be paid to a proper formation of analyte-matrix co-crystals, which can be hindered by the presence of silica gel [50]. Therefore, other desorption ionization techniques, such as DESI (desorption electrospray ionization) [51,52], or SALDI (surface-assisted laser desorption ionization) [53], have also been employed in MS(I) analysis of TLC plates. However, TLC-MALDI-MS(I) has successfully been used for analysis of pharmaceuticals [54], drugs of abuse [55], lipids [50,56], glycolipids [57,58] oligosaccharides [59], and also small peptides [60]. Ivleva et al. [57,58] investigated direct MALDI analysis of TLC plates with an FTMS instrument equipped with a vibrationally cooled MALDI ion source. The vibrational cooling during ion formation in the MALDI plume prevented fragmentation of labile gangliosides species. As a result, straightforward identification of individual gangliosides was achieved even within incompletely spatially resolved TLC bands.

Furthermore, the potential of IR-MALDI for TLC read-out was addressed by several research groups [50,54]. As reported by Rohlfing et al. [50], IR-lasers are capable of penetrating much deeper into a TLC plate in comparison to UV-lasers. As a consequence, analytes from the deeper silica gel layers are efficiently released. Since IR-MALDI enables the usage of a wider range of matrices, e.g. glycerol, problems with decent analyte-matrix co-crystals formation are ameliorated [50].

The single bands on a TLC plate often consist of more than one molecular species. By MSI of the plate, the separation resolution is increased over the conventional read-out and the individual analytes within one band are differentiated. This is shown in Fig. 5 where lipid species were separated on a TLC plate, which was subsequently imaged using MALDI-MS. Kuwayama et al. [55] demonstrated the use of TLC-MALDI-MSI for analysis of drugs of abuse in biological samples. They alleviated the need for lengthy sample preparation by TLC separation of 11 psychotropic compounds. They detected the drugs of abuse and visualized the spots on a TLC plate with MALDI-MSI. MALDI-MSI is demonstrated to be capable of providing a far more robust molecular identification when compared to the information based on retention factor only. The application of tandem MS in these MALDI-MSI experiments increases the identification reliability.

3.2. *Blotting: adding the z-dimension*

Generally, during a blotting experiment the analytes, previously separated in one or two dimensions, are further detached from the separating environment in the vertical direction. Nevertheless, separation is not always necessary since a solid substrate such as tissue section can be blotted directly. Blotting augments sensitivity of MS detection and further lowers sample complexity. It also facilitates sample transfer to a MALDI mass spectrometer by capturing the analytes of interest onto a membrane surface, thus encompassing the fragile gels and non-inert silica plates. The overall advantages of the membranes used in MALDI-MS analysis have already been summarized in Section 2.1 of this review.

3.2.1. *Blotting TLC plates*

Blotting helps to prevent the band broadening during direct matrix application on a TLC plate [48]. Gusev and co-workers [61,62] introduced an alternative methodology based on crystallization of matrix on an inert substrate and subsequent imprint of the matrix layer on top of the TLC plate. In other work, blotting of TLC plates improved IHC based detection of glycosphingolipids

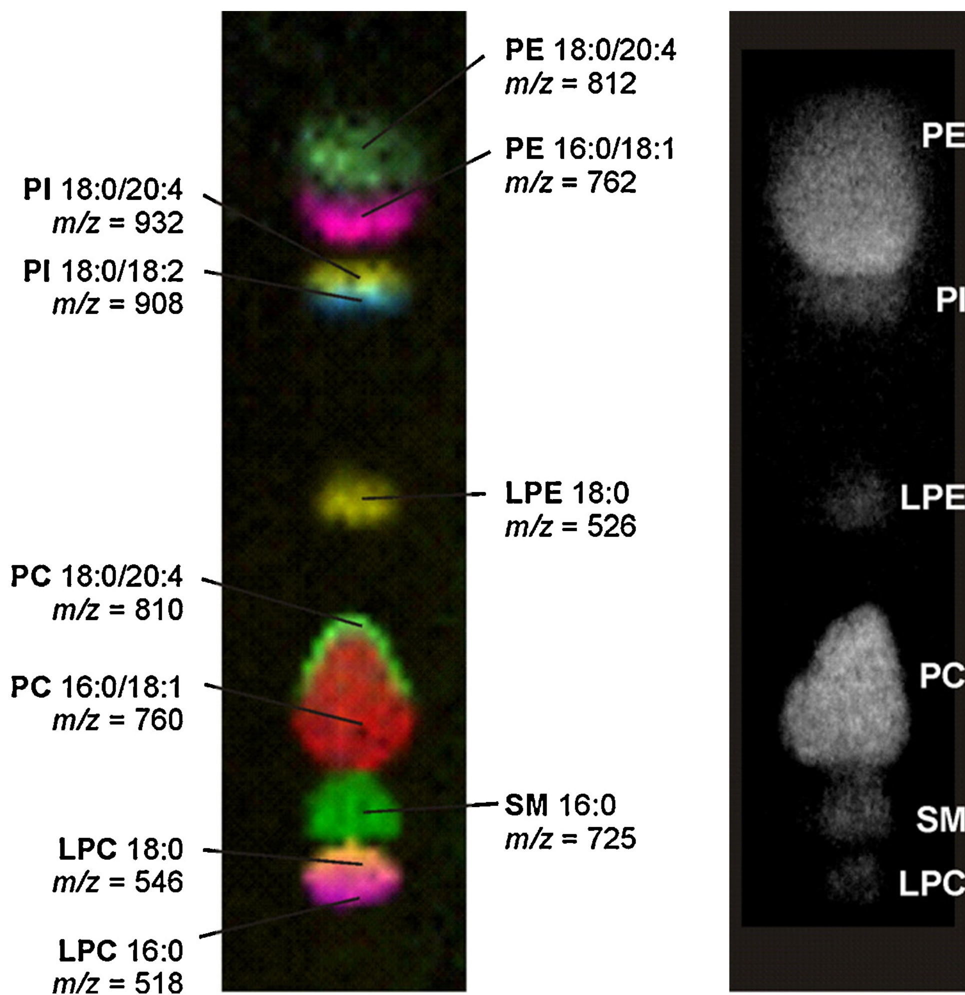

Fig. 5. Lipids from hens' egg yolk separated by thin layer chromatography (TLC) and visualized by matrix assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) (left panel) and by densitometry (right panel). MSI clearly separated lipid species within individual lipid classes (PE, phosphoethanolamine; PI, phosphoinositol; LPE, lysophosphoethanolamine; PC, phosphocholine; SM, sphingomyelin; LPC, lysophosphocholine), whereas the conventional approach could tell apart only the particular lipid classes. MS imaged two different spots for most of the lipid classes and differentiates lipids with longer and shorter fatty acyl residues. MALDI-MSI increased separation resolution of TLC and provided with deeper understanding on the mixture composition. Figure reprinted with permission from reference [47]. Copyright 2010 Elsevier.

(GSL) and phospholipids separated in a TLC experiment [63]. The first blotting experiments involved transfer of GSL onto a nitrocellulose membrane [64]. Since the reproducibility and efficiency of that experiment were low, Taki and co-workers [65] tested the suitability of other polymeric membranes, and finally substituted the nitrocellulose-based probe with a PVDF membrane. In later work, they replaced the IHC detection with MS [66]: the bands of blotted GSL were cut out of the PVDF membrane and analyzed with SIMS. Kasama et al. [67] demonstrated the TLC-blot-SIMS method for screening GSL composition of rat mammary tumour cell lines with different metastatic potentials. Guittard et al. [68] adopted the blotting protocol of Taki's group [65] and compared direct MALDI analysis of TLC plates and TLC blots. The group reported on better spectral quality of the membrane-fixed analytes, demonstrating higher sensitivity, higher signal-to-noise ratio and better mass resolution.

Gusev et al. [61] reported on imaging of TLC using MALDI-MS. Their work was based on microprobe mode MSI obtaining contour-plots. The pioneering work on MALDI-MSI of TLC blots was done by Taki and co-workers [63,69]. Their blotting protocol is performed as follows: a developed. TLC plate is dipped into a blotting solution for several tens of seconds and placed on a glass plate. Subsequently, a PVDF membrane, a Teflon membrane and a glass fibre sheet paper are placed on top of the TLC plate, followed by pressure from an iron heated to 180 °C for 10–30 s. The benefits of Taki's TLC-MALDI-Blot-MSI method were demonstrated in several applications [49,70,71]. Similarly to direct MSI of TLC plates, imaging of the blot membranes increases the separation resolution. For instance, the combination of TLC and MSI of the blot membranes helped to distinguish two molecular species of GSL GM1, which created only one band on the TLC plate when detected by primuline based staining [49]. In another publication the glycolipidome of patients with Alzheimer's and Parkinson's disease was compared to control samples [71]. Significant differences in lipid composition within the hippocampal region were reported. This application demonstrated the complementarity between MSI and separation techniques: the brain tissue extracts were separated using TLC and the lipid species were further separated from the plates in the third dimension by employment of a blotting step. High separation resolution was achieved and brain lipid composition was studied by MSI of the blots obtained (see Fig. 3).

3.2.2. *Blotting gels*

Most of the methods for protein identification from gels take the advantage of transfer of the separated proteins onto a more robust polymeric membrane [72]. The protein spots are cut out of the membrane and homogenized for identification by Edman degradation, hence the information on protein position within the

gel/membrane is lost. On the contrary, during IHC based detection the imprint of proteins on the membrane remains intact, hence the proteins are identified while their spatial distribution is preserved. The immunoassays, however, suffer from significant drawbacks: dependency on existence of specific antibodies and limited possibility of simultaneous detection of multiple analytes. Those pitfalls are successfully ameliorated by MALDI-MSI. In general, MALDI-MS(I) analysis of proteins blotted onto a membrane surface is more feasible when compared to gels owing to the higher stability of membranes. Moreover, the proteins are transferred in a highly purified state free of contaminants such as SDS, salts or buffers [18,73,74], hence the ionization suppression effects are reduced.

MALDI-MSI of 2D-GE separated and subsequently blotted proteins has not been established for routine analysis yet (except of the molecular scanner tool discussed in Section 4.2). Vaezzadah and co-workers [16] reported on MSI of tryptic peptides blotted onto a polymeric membrane after IEF. Their approach allows a MSI based preview of a proteome to be obtained, and provides information such as relative concentration of proteins, presence of contaminants, and overall complexity of the sample. Based on this knowledge, strategies for further proteome analysis can be designed [16].

A number of MALDI-MS applications for analysis of blot-transferred proteins have been described and reviewed extensively [18,72–76]. Some authors paid attention to the comparison of UV- and IR-MALDI for on-membrane captured proteins analysis and the suitability of membrane types [18,75,76]. It was found out, that while proteins on sample support membranes are well desorbed with UV-MALDI, the spectral quality of blotted proteins is significantly lower [76], and IR-MALDI becomes more suitable [18,74,75]. Further, several matrix application methods to avoid the protein bands spreading were tested. Strupat et al. [18], for instance, recommended matrix application by soaking the wet membrane into an aqueous matrix solution rather than wait until the membrane becomes dry and then rewetting with organic solvents during matrix application. The organic solvents relocate the proteins within the membrane and an accurate spatial information is lost [74]. Another possible solution of band spreading prevention might be blotting the gel on a membrane already precoated with matrix as previously performed for tissue sections [77]. Application of this approach to gel-separated proteins however, is yet to be demonstrated.

3.2.3. Blotting tissue sections

The concept of blotting tissue surfaces to obtain a mirror molecular image was introduced in the very first paper on MALDI-MSI [8]. Caprioli and co-workers [8] acquired tissue imprints by contacting the exposed cells of a freshly cut tissue with various targets: cellulose membrane, surface covered with C18 beads and stainless steel probe. Blotting of the tissue sections led to selective separation/extraction of proteins and peptides from interfering compounds such as salts and lipids. Chaurand et al. [19] demonstrated successful MALDI-MS profiling of tissue transfer blots on a conductive carbon filled PE membrane. The chemical properties of the membrane allowed for a protein separation/extraction from the tissue surface and for a subsequent water based washing. Vidová et al. [78] demonstrated transfer of lipids by blotting tissue surface onto a nano-assisted laser desorption ionization (NALDI) target followed by matrix-free NALDI-MSI. Bunch et al. [77] demonstrated the applicability of the blotting approach for low-molecular compounds. They used a matrix (CHCA) precoated cellulose membrane to transfer ketoconazol (observed at m/z 531.19) by blotting a medicinal shampoo treated porcine skin sample. The group investigated also a quantitative approach of their blotting methodology, obtaining a good linearity when a matrix peak was employed to normalize the signal.

Acquisition of a positive imprint of a tissue section represents a separation step where only the z-dimension is employed. As demonstrated, blotting leads to effective separation of compounds of interest, thus decreasing the tissue complexity and reducing ionization suppression. The imprinted areas can be washed by applying rinsing solutions. If performed carefully, spatial information is preserved [8,77]. Special attention also has to be paid to timing of the blotting to avoid tissue dehydration [19].

Another application of tissue blotting was published by Amstalden van Hove et al. [79]. The group presented an alternative method for tissue washing based on close contact of a tissue section with a wetted fibre-free paper. Separation of salts from the sample surface was demonstrated. The advantage is that this approach allows for washing of delicate tissues or samples, such as those mounted with double-sided tape that cannot be washed by conventional methods. The method also allows for washing a selected area of a tissue sample exclusively.

4. One step blotting and digestion

4.1. Protein identification strategies for MS

Determination of molecular mass of an intact protein is not sufficient for its unambiguous identification [75]. MS strategies for protein identification have been reviewed in the past [20,28,80,81] and a concise review on protein identification using MSI was recently published by Mascini and Heeren [82]. Two strategies are distinguished based on the absence or presence of a protein pre-digestion step within the MSI workflow, i.e. top-down and bottom-up approaches [82,83]. In a top-down experiment, intact proteins are directly analyzed in a mass spectrometer. By subsequent MS/MS analysis, the proteins are fragmented according to well-described patterns, which allow for protein identification using specific databases [13,82]. The bottom-up approach requires a protein digestion preceding MSI. The emerging peptides are analyzed by MS and the subsequent identification process is either based on peptide-mass-fingerprinting (PMF) or on MS/MS analysis [82]. The top-down MS strategy is limited by the performance of the currently available instrumentation. For instance, when using a MALDI-TOF mass spectrometer it is usually impossible to analyze high-molecular mass proteins (M_m > 30 kDa) [13,82,84]. The mass dependent sensitivity drop-off causes a loss of signal for the larger proteins [13]. Protein digestion brings the proteins into a detectable mass range with sufficient sensitivity [13].

The protein digestion for a bottom-up approach can be performed before the gel separation [16], directly in gel after 2D-GE [85], on a tissue section before a MSI experiment [86], as well as on a support membrane after gel/tissue blotting [19,75] or sample deposition [24]. The in situ digestion approach limits additional sample handling and therefore appears to be the most convenient. However, presence of significant interfering background signal is stressed especially for tissue samples [82]. A strategy for lowering complexity of digested samples while preserving the protein spatial distribution within the gel/membrane/tissue has been presented in the literature. The approach is known as the molecular scanner and is well suited for MALDI-MSI analysis.

4.2. Molecular scanner

The principle of molecular scanner was introduced by Hochstrasser's group in Geneva in 1991 [87] as a tool for automated high-throughput proteome research, and since then a number of papers have been published on the topic [87–92]. The concept is based on three adjacent layers: a gel with separated proteins

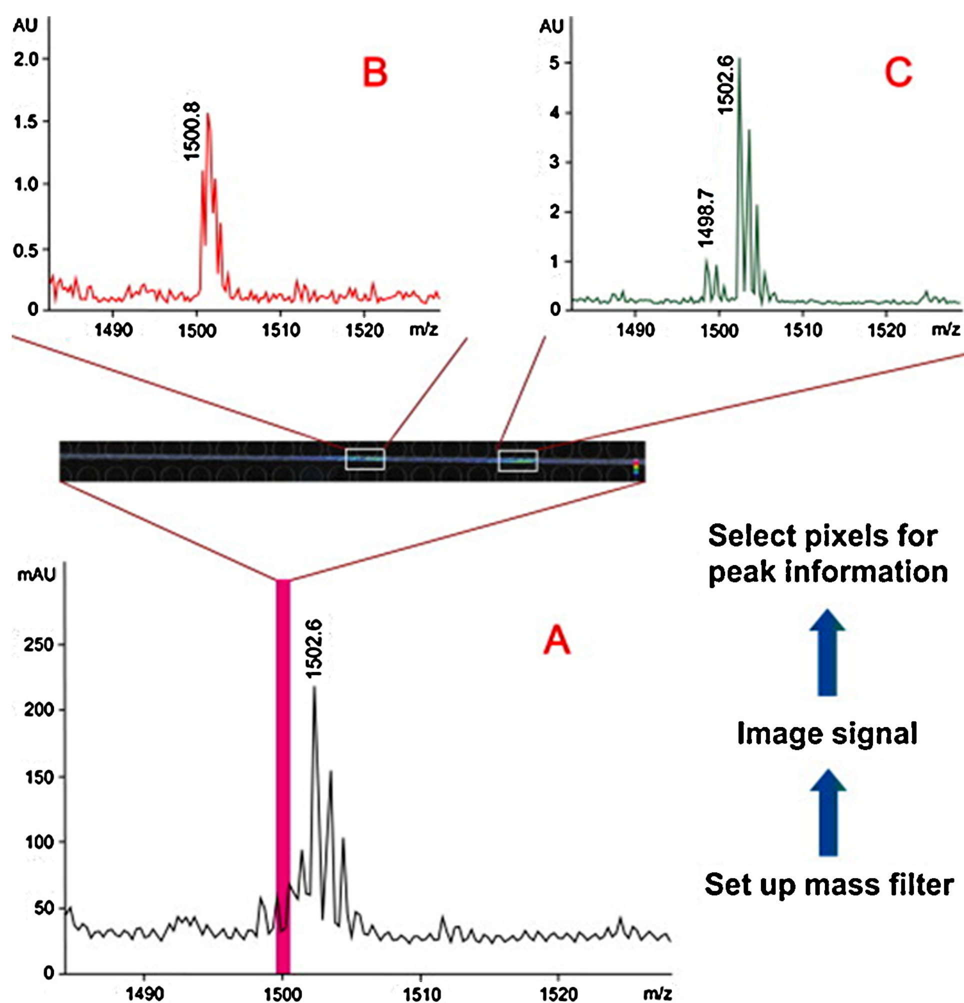

Fig. 6. Workflow for complementary use of liquid chromatography (LC) separation and mass spectrometry imaging (MSI). (A) MS full scan of a bovine serum albumin tryptic peptide (*m*/*z* 1502.6) showing the mass range from *m*/*z* 1490 to *m*/*z* 1520. Low abundant *m*/*z* are suppressed by the main signal at 1502.6; 1 Da wide selected mass filter is represented by the pink strip. The corresponding image for the selected mass filter signal is enlarged, and highlighted in the white boxes, there are two individual peptides with different retention times. (B and C) Mass spectrum of a particular region of interest corresponding to the separated peptides. The peaks at *m*/*z* 1500.8 and 1498.7 are now observed with better resolution. The three-step approach (1) select mass filter, (2) obtain image, and (3) extract ions from pixel/region of interest, can also be used when looking for unknown analytes within complex mixtures by screening the mass filter across the entire mass spectrum. Figure reprinted with permission from reference [97]. Copyright 2013 Elsevier.

or a tissue section, a hydrophilic membrane with covalently bound trypsin, and a polymeric (PVDF) capture membrane [83]. By applying an electric field, the proteins migrate through the enzymatic membrane and resulting proteolytic peptides are captured on a collection membrane. In a subsequent step this capture membrane is imaged using MS and the final intensity based plot is reconstructed. Several software packages were developed for the evaluation of the 2D proteome images. Müller et al. [91] reported in detail on visualization and analysis of the molecular scanner generated peptide mass spectra. Data generation and analysis was thoroughly described [90,92].

The above-described protocol is known as "one step digestion transfer" (OSDT). Another assessment includes digest of the proteins directly in the gel. This "parallel in-gel digestion" approach leads to more efficient digestion and is particularly suitable for proteins which are too large to be transferred through the enzymatic membrane. It suffers, however, from loss of mass resolution for low molecular mass peptides [88]. The best results were obtained when the proteins were first in-gel pre-digested with a small amount of trypsin and followed by standard OSDT ("double-parallel digestion" approach) [88].

One of the general limitations of the molecular scanner is that some proteins are more easily extracted from the gel/tissue surface than the others, which results in only a partial protein content analysis [13,84]. The spatial resolution of a scanning experiment is also a limiting factor. As the whole protein band cannot be concentrated into a single spot on the collecting membrane, the sensitivity of the method is limited [89]. This drawback can be overcome by the MSI approach, especially by the employment of MSI in microscopic mode [83]. Luxembourg and co-workers [83] separated proteins in one dimension and performed the molecular scanner experiment. The collecting PVDF membrane was subsequently covered with matrix and a gold layer to avoid charging effects. The prepared membrane was imaged using MALDI-MSI in microscopic mode and allowed for high spatial resolution imaging of the captured peptides. Rohner et al. [84] reported on application of the molecular scanner approach for analysis of tissue sections by the means of MSI. The capture membrane was MS imaged to visualize proteolytic peptides generated from the tissue surface. The preliminary results showed preserved spatial information and reduced ionization suppression [84]. The working protocol, however, needs additional optimization.

Box 1: Microprobe mode vs. microscope mode MSI
Microprobe mode MSI is more frequently employed approach of MSI [13]. The focused laser beam rasters the sample surface and a mass spectrum is collected at each raster point. The spatial resolution is determined by the laser spot diameter [13,93]. The final image is defined by the positions of mass spectra collected and intensity of a particular *m/z* throughout these spectra. The images can be represented in a form of colour scaled density maps, 3D intensity profiles [61,90] or contour-plots [61,74]. In **microscope mode** an unfocused laser illuminates a larger area of the sample surface. The ion optics preserves the ion spatial distribution of the desorbed ions until they reach a position sensitive detector [94]. Microscope mode MSI generates highly resolved ion images with spatial resolution up to 4 μm within the laser spot that has a diameter of ~ 200 μm [13,93]. Large data sets are generated in microscope mode MSI due to the high spatial resolution involved. It is advantageous to combine the microprobe mode for screening with subsequent microscope mode high spatial resolution of targeted compounds [83].

5. Column and capillary separations

5.1. Direct MALDI interface

Capillary electrophoretic (CE) and liquid chromatographic (LC) separation methods are nowadays commonly coupled to mass spectrometers, which allow for highly sensitive and selective identification of the analytes. The interface of choice for coupling LC and CE to MS is ESI since it allows for direct introduction of liquid samples into a mass spectrometer, i.e. it allows for a convenient on-line coupling. However, in comparison to ESI, MALDI has several important benefits, some of which have been already addressed in the previous text, i.e. it is less sensitive to the presence of contaminants, it requires significantly smaller amounts of sample, offers faster analysis and thus higher throughput, and provides simpler mass spectra since singly charged molecular ions are created [14,29,44]. The possibility of MS/MS analysis is a prerequisite for an accurate analyte identification. In an on-line LC-ESI approach MS/MS of a selected *m*/*z* must be performed within the elution time of the analyte. MALDI can overcome this problem since it enables re-analyses of a capture spot until the sample is completely used [14]. The advantage of CE- or LC-MALDI becomes obvious when analyzing very complex mixtures of protein and peptide mixtures that are often too complex for an unambiguous identification [29].

Several interfaces of coupling column and capillary separation to MALDI-MS have been described in the literature and some of them have been reviewed by Gusev [44]. The simplest approach is an off-line fraction collection of the eluent with a subsequent spotting of sample array on a MALDI target and addition of matrix. However, on-line coupling significantly improves the interface [29,95]. It can be achieved either by deposition of discrete fraction droplets [29,95], or by deposition of a continuous flow of the eluent. The eluent flow is either coupled to a flow of matrix, so that both solutions are deposited on a MALDI target simultaneously [96,97], or utilizes MALDI targets that are precoated with matrix [14,29,30].

Independently of the CE/LC-MALDI interface, the MALDI experiment itself is typically achieved by analysis of discrete, individual spots. However, this approach is ineffective and time-consuming, and detailed information on the retention times of particular analytes is lost. On the contrary, the nature of MSI makes it compatible with coupling to micro-scale separation methods while spatially preserving the retention time information, and significantly easing the data collection [97]. An image of an ion with a particular *m*/*z* can be constructed from the deposited eluent with MSI as a detection method. The position of this ion now represents its retention time within the separation process [97].

For instance Zhang et al. [97] reported on the advantageous LC-MALDI-MSI combination. They described separation of neuropeptide mixtures, continuous flow deposition of the eluent on a moving MALDI target with subsequent MSI analysis of the deposited eluent lines. Their practical workflow of the image-based analysis of a mass spectrum with complex and low abundant peaks is shown in Fig. 6. Their approach enabled identification of a significantly higher amount of peptides when compared to traditional fraction collection followed by a spot-by-spot MALDI analysis (81% vs. 66% of total protein sequence for MSI and spot-by-spot analysis, respectively). In another publication, Zhang et al. [15] utilized MALDI-MSI in a robust off-line platform coupled with pressure enhanced capillary electrophoresis. 2D separation combining LC and CE with subsequent MALDI-MSI analysis was evaluated for studies of neuropeptide mixtures [98]. It was the first, and to-date only, combination of 2D column/capillary separation with MSI [98].

5.2. Validation and corroboration of MSI results

Ionization suppression represents a particular problem in quantitative MSI (qMSI), which aspires to become a fully established quantitation method for e.g. pharmacokinetics and drug stability studies. Importantly, the degree of ionization suppression for a single analyte is tissue type dependant. This fact complicates qMSI performed in whole body tissue sections, where multiple tissue types are present. The imperfections in the matrix layer homogeneity as well as MALDI shot-to-shot variability account for further bottlenecks. Several approaches of alleviating the MALDI-qMSI pitfalls have been recommended and/or tested, and publications reporting on successful qMSI can be found in the literature [99–102] and some of the works referred to therein. Pirman et al. reported on the use of isotopically labelled internal standards for MSI [103]. However, to validate results obtained by qMSI, well-established quantitative techniques such as LC-MS/MS [99–101] or QWBA [99] are still needed. These techniques lack some of the advantages of qMSI, such as label-free spatial information but provide reliable quantitative data. Beside the corroboration in quantitative research, LC-MS/MS is also used to validate qualitative information obtained by MSI. This approach became useful for instance during search for new natural products [104] and biomarkers [105], or for confirmation of peptide identification through accurate mass analysis [106].

6. Conclusions and future perspectives

In this review, we have discussed the benefits of strategies that combine analytical MALDI-MSI and various separation techniques. Both, MALDI-MSI and separation techniques provide an analytical scientist with information on the molecular composition of a sample. The essential contribution of MSI is the spatially correlated molecular information. The separation techniques have the ability to significantly reduce ionization suppression, hence improve ionization of low-abundant molecular species. A comprehensive report on sample composition is obtained by their combination in a single analytical approach. However, there is still room for improvements.

For instance, the blotting technique, introduced at the dawn of MALDI-MSI, has remained relatively unexplored, especially with respect to the tissue sample analysis. As demonstrated in several applications the blotting process preserves the spatial distribution of the studied molecules when performed carefully. It is known that different classes of compounds vary in their ability to be adsorbed

onto diverse polymeric materials. Moreover, the z-dimension based separations could be performed selectively by further derivatization and/or functionalization of the polymeric probes. As a result only particular analytes would be separated from a pool of the compounds present on a tissue surface.

Another set of improvements can be introduced with the new ionization techniques. Dramatically fast developing ambient ionization techniques, such as laser ablation electrospray ionization, offer the advantage of analysis of vacuum incompatible samples, and of little to no need for sample preparation steps. The problem of bands broadening upon matrix application would therefore be overcome. Furthermore, ambient ionization techniques would allow for direct analysis of gels with separated proteins without significant problems with gel in-vacuum stability.

Discovery or development of a universal analytical method capable of unbiased and highly sensitive detection of all molecular classes within one experiment is still far from achieved. Until its introduction, the most relevant information on a sample composition will be obtained based on combinations of existing complementary analytical methods, which are being improved constantly.

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