9
Small-Molecule Desorption/Ionization Mass Analysis

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9.1
Introduction

At its advent, the application of MALDI mass spectrometry (MS) to low-molecular mass (LMM) compounds (so-called "small molecules") seemed quite unlikely, due to saturation by matrix ions signals below 500 Da. Although MALDI-MS has literally transformed the analysis of high-molecular weight biomolecules, its application to LMM compounds has lagged behind. The advantages of MALDI for soft and efficient ionization of various fragile and non-volatile samples should, in theory, circumvent the problems previously exhibited by laser mass spectrometry, including severe fragmentation for even low-molecular mass organic molecules, the need for high laser energy, and reduced ionization efficiency [1–3]. Other advantages of MALDI, including tolerance for contamination and buffers, uncomplicated spectra as most ions are singly charged ions, very high absolute sensitivity [4], rapid analysis compared to electrospray ionization mass spectrometry (ESI-MS), and relatively simple instrumentation, serve to reinforce a logical rationale to utilize this technique in the lower mass arena.

Until recently, the use of MALDI to characterize small molecules has been "suppressed" by multiple factors, including the low resolution of first-generation linear TOF-MALDI instruments, matrix ion interference and detector saturation in the low mass range, complex coupling of MALDI with on-line techniques such as liquid and planar chromatography, and – most importantly – strong competition from ESI-MS technique in general. As a result, most mass spectrometric practitioners have for some time considered "conventional" MALDI to be inappropriate or even useless for the determination of LMM compounds. However, interest in the application of MALDI to small molecules has continued to grow over the past decade [5]. Improvements in TOF systems – and specifically the utilization of delayed extraction (time lag focusing) – has led in turn to remarkable improvements in MALDI-TOF resolution [6,7]. With recent technological advances in MS instrumentation, a range of mass spectrometers has been interfaced with MALDI sources, the most popular and impacting to date being tandem mass spectrometers. MALDI-MS/MS approaches have been driven by advances in two

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key instrument components: (i) high-repetition lasers which can be satisfactorily used with existing triple quadrupole instruments; or (ii) atmospheric pressure (AP) MALDI sources (see Chapter 1), which can be coupled to a range of mass analyzers including ion traps, TOFs, and quadrupole instruments. As will be described in greater detail in this chapter, the progress achieved in the use of structured solid support materials, such as those used in Desorption Ionization on Silicon (DIOS), is also increasing the inherent utility of MALDI for small-molecule analysis. In addition, a continued demand for high-throughput methods in drug discovery and biotechnology, as well as the analysis of complex mixtures in high-salt matrices and buffers, has regenerated efforts to utilize the full power of MALDI-MS over the entire mass range of interest.

This chapter will explore the qualitative and quantitative investigations of MALDI-MS for molecules with a mass less than 1500 Da. As an exhaustive exploration of studies involving any molecular ions detected at 1500 Da or less could yield thousands of articles, we have concentrated on highlighting recent relevant studies that focus on LMM compounds. More detailed information on LMM biomolecules and polymers can be found in Chapters 3–8. In addition to exploring alternative sample preparation approaches, coupling MALDI to various separation techniques will be examined as mechanisms to strengthen the utility of MALDI in the low mass range.

9.2
Matrix Choices for Small-Molecule MALDI

The successful application of MALDI in the analysis of a wide variety of molecules lies in its ability to generate intact ions of thermally labile molecules using a UV-absorbing matrix. A variety of compounds has been used as MALDI matrices, depending on the application. In general, a good MALDI matrix should have the following properties: (i) the ability to absorb at the wavelength of the laser used; (ii) the solubility of the matrix with the analyte; and (iii) the ability to transfer protons during the ionization process. As most of these matrices have molecular weights below 500 Da, the presence of matrix-related ions interferes with LMM analyte detection. Thus, careful choice of the matrix is crucial to the successful application of MALDI to small-molecule analysis. Essentially, if a MALDI matrix provides efficient ionization, minimal or controllable fragmentation and exhibits lack of mass interferences, it can be used for the analysis of LMM compounds. Those matrices found to be useful for small- molecule analysis are discussed in the following sections.

9.2.1
Organic Matrices

The most widely used conventional matrices in the analysis of LMM compounds are crystalline organic molecules that have strong UV absorbance. Based on their chemical structures, two main classes of these conventional matrices are used in MALDI: cinnamic acid derivatives; and aromatic carbonyl derivatives [8]. The use of cinnamic acid derivatives – in particular, α-cyano-4-hydroxycinnamic acid (α-CHCA) and aromatic carbonyl derivatives such as 2,5-dihydroxy benzoic acid (DHB) – has proven to be effective in the analysis of a variety of small molecules [9–11]. Both α-CHCA and DHB yields spectra with high signal-to-noise (S/N) ratio and resolution. The use of these matrices in the analysis of LMM relies heavily on the matrix-to-analyte molar ratio. Unlike MALDI analysis of high-molecular weight compounds, which require an optimal ratio in the order of 10^3 to $10^5:1$ [12], the matrix-to-analyte molar ratio is much lower for LMM compounds (10^{-1} to $10^3:1$) [10,13].

As most MALDI applications rely on solid (crystalline) organic matrices, which may create considerable background in the low mass range and complicate the characterization of LMM compounds, a few organic crystalline matrices (e.g., 2-hydroxy-1-naphthoic acid [14]) have been introduced specifically to address the characterization of LMM samples such as peptides and porphyrins. One clever approach to circumvent matrix interference was to use a higher molecular weight matrix, which does not interfere in the low-mass region. To this end, some porphyrins have been used as MALDI matrices [15–17].

The use of multicomponent matrices with accelerated sample drying along with a modified instrumental set-up was also considered when preparing samples for quantitative MALDI analysis. This method has been shown to improve the measured relative standard deviation (RSD) to better than 10% of analyte to internal standard (IS) intensity ratio [18]. Both, signal reproducibility and precision of the standard curve slope were improved by a factor of two when a DHB/fucose/5-methoxysalicylic acid multicomponent matrix was used compared to DHB alone. Similar results were observed when a ferulic acid/fucose multicomponent matrix was used compared to the use of ferulic acid alone.

9.2.2
Inorganic Matrices

An alternative approach to organic crystalline matrices involves the use of inorganic matrices. This was originally introduced by Tanaka et al. in 1988, and utilizes ultrafine 30-nm diameter cobalt powder suspended in glycerol [19]. This approach was applied to the analysis of polyethylene glycol (PEG) 200 and methyl stearate using metal or metal oxide powders suspended in liquid paraffin or glycerol [20]. Paraffin exhibited a lower background than glycerol and, interestingly, the analytes were cationized with Na^+ or K^+ ions, not the metal species suspended in the liquid matrix. Unfortunately, higher laser fluences were needed for ionization, which caused increased analyte fragmentation. Despite this, the investigators still observed the “sweet spot” effect with these suspension-type matrices and problems with the vertically mounted sample holder, which caused the sample physically to shift to the bottom of the holder.

Sunner et al. have introduced the surface-assisted desorption/ionization (SALDI) technique, which utilizes graphite particles suspended in a mixture of glycerol, sucrose, and methanol [21]. An optimized sample preparation involved evaporation of the graphite suspension onto a solid substrate, followed by spotting the analyte solution onto the suspension. It is important to note that the size of graphite particles is between 2 and 150 μm, or a few orders of magnitude larger than those used by Tanaka et al. [19]. The applicability of the SALDI method was demonstrated for small organic molecules and peptides, and the results obtained for LMM analytes were similar to those obtained by fast atom bombardment (FAB) MS. These authors noted that SALDI mass spectra exhibit a relatively low chemical background, and are well suited to the characterization of LMM compounds. Graphite powder was originally used for SALDI, but at a later stage a micrometer-sized activated carbon powder was found to provide even better results.

The use of surfactant additive to a SALDI matrix system was shown to enhance SALDI sensitivity [22]. A marked improvement in signal intensities of small organic molecules was observed using appropriate amounts of charged surfactants with acidic characteristics. A detection limit of 100 pg was achieved in the analysis of methylephedrine with a 0.5 M *p*-toluenensulfonic acid (PTSA) added to the SALDI sample preparation. It was suggested that PTSA not only acts as a proton source for the analytes; rather, its interaction with the protonated analyte species also facilitates their migration to the surface, thereby enhancing the signal intensity. Thus, the ionic interaction between surfactant and the analyte was proposed as a possible mechanism for the demonstrated surfactant-enhancing effect. In a subsequent study, use of the surfactant additive was extended in the TLC-SALDI analysis of porphines [23]. In order to obtain homogeneous carbon particle deposition onto the TLC plate, carbon particles ablated from a 1-mm pencil line drawn along the track of the developing sample before the separation were used in the SALDI matrix system. This combination of uniform analyte distribution and the addition of PTSA to the SALDI matrix system increased the S/N ratio of the analyte peaks and increased the sensitivity 50-fold compared to the conventional TLC-SALDI method. In another study, the use of surfactant-modified carbon powder as solid-phase extraction (SPE) sorbents and carbon particles in the SALDI matrix system demonstrated an increase in sensitivity of the SPE SALDI analysis of trace organic compounds in water [24]. In SPE SALDI, the samples are passed through the SPE cartridge, which is subsequently dried and the trace organic compounds adsorbed onto the surfactant-modified SPE sorbent are analyzed directly. In this way, detection limits of trace organic compounds as low as 25 ppt from 100-mL water samples were obtained.

In addition to the use of graphite in SALDI, alternative carbon particles such as carbon nanotubes (CNTs) have been shown to be effective inorganic matrices for MALDI analysis. CNTs obtained from coal by arc discharge were used as MALDI matrix in the analysis of small peptides, organic molecules, β-cyclodextrin, and small proteins [25]. The matrix is prepared by suspending the CNTs in ethanol, followed by sonication for a few minutes to fully disperse them. Sample preparation involves the deposition and drying of the CNT matrix on the MALDI probe, followed by deposition of the analyte of interest. Mass spectra obtained with CNT matrices were characterized by alkali metal ion adducts of the analyte peaks, with few or no background ions. Interestingly, CNTs require a lower laser fluence for the desorption/ionization of peptides, and this results in a higher detection sensitivity (low femtomole range) than with conventional organic matrices. In order to reduce alkali metal ion adducts and to observe more protonated species, citrate buffer – which serves as a proton source as well as an alkali metal ion-chelating reagent – is added to the CNT matrix system [26]. An inherent problem with CNTs, however, is their tendency to fly off from the target plate when subjected to a laser pulse; this can result in contamination of the ion source, time-limited analyte signals, and a time-consuming search for "sweet spots". Ren et al. described a method to overcome this limitation by immobilizing the CNTs with polyurethane adhesive for more prolonged analysis [27]; such immobilization of the CNTs did not affect their properties as matrix. As shown in Figure 9.1, immobilized CNTs as matrix were successfully applied to monitor glucose in urine samples from healthy and diabetic patients. In a recent follow-up study, the same group used chemically modified CNTs in the analysis of neutral carbohydrates, small peptides, and proteins [28]. Chemically modified CNTs terminated with hydroxyl and carboxyl groups were prepared by treating oxidized CNTs with dilute nitric acid. This modification resulted in an increased surface polarity, which provided modified CNTs with a better shot-to-shot reproducibility than for unmodified CNTs. An analysis of simple neutral carbohydrates with mass <500 Da showed analyte peaks with high S/N ratios and detection sensitivity in the low femtomole range.

9.2.3
Liquid Matrices

Some unfortunate drawbacks of using crystalline matrices in MALDI analysis are poor shot-to-shot and sample-to-sample signal reproducibility and "sweet-spot" phenomena. An apparent alternative to crystalline UV MALDI matrices is the utilization of liquid matrices [29–31]; these provide a long-lasting signal, do not have "sweet-spots", exhibit higher signal reproducibility, and are miscible with both polar and nonpolar analytes. A number of organic UV-absorbing additives were tested to widen the applicability range of liquid matrices [32]. However, the use of liquid matrices is still associated with low mass resolution, high chemical background, potential instrument contamination and poor ionization efficiency.

The SALDI approach with two-phase matrices was investigated by Zenobi and coworkers [33]. In addition to glycerol, a few liquid matrices [e.g., 3-nitrobenzyl alcohol (NBA), nitrophenyl octyl ether (NPOE) and thioglycerol] were successfully used. Detection sensitivity (5–50 fmol) was comparable to that obtained by conventional crystalline matrices. In a follow-up report [34], it was also demonstrated that the selection of liquid phases can be guided by criteria developed previously for FAB – that is, the more-acidic glycerol promotes protonization whereas the more-basic diethanolamine favors deprotonization. Minimal background (chemical noise) was observed in the low mass range.

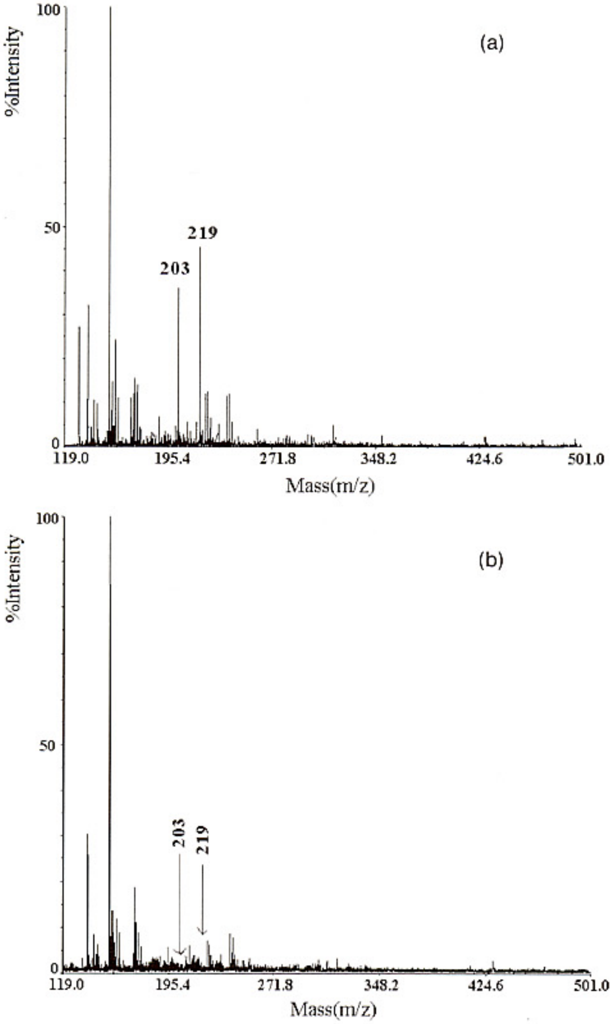


Fig. 9.1 MALDI-TOF mass spectra for urine sample of: (a) a diabetic patient; and (b) a healthy person. Unlabeled peaks arose from a normal urine sample. Ion peaks at *m/z* 203 and 219 correspond to urinary glucose. Ion peaks for urinary glucose were observed in the spectrum for the diabetic patient, while there was no visible glucose signal on the spectrum for the healthy person. Reprinted by permission of Elsevier from Ref. [27]; © American Society for Mass Spectrometry, 2005.

9.2.4
Matrix-Free Approaches

The introduction of a matrix-free desorption/ionization on electrochemically etched silicon surfaces has allowed the analysis of analytes with mass as low as 100 Da [35,36]. In desorption/ionization on silicon mass spectrometry (DIOS-MS), analytes are deposited onto the porous silicon (pSi) surface and desorbed/ionized by the irradiation of a UV-emitting laser. The morphology of pSi provides a scaffold for retaining solvent and analyte molecules, and its UV absorptivity affords a mechanism for transfer of the laser energy to the analyte. This unique combination of characteristics allows DIOS to be useful for a large variety of biomolecules including peptides, carbohydrates, and small organic compounds of various types. Unlike other direct, matrix-free desorption techniques, DIOS enables desorption/ionization of LMM compounds with little or no analyte fragmentation. Recently, Trauger et al. showed that silylation of the oxidized pSi using a variety of commercially available silylating agents results in a much-improved DIOS-MS performance [37]. A dramatic improvement in sensitivity, significant advantages in shelf-life, ease of modification, and analyte specificity was demonstrated from the DIOS analysis from the silylated pSi surface. Figure 9.2 illustrates some examples of mass spectra obtained using the silylated pSi surfaces. Note that minimal background is observed in the low mass range. DIOS and MALDI post-source decay (PSD) have also been compared to ESI data (see Fig. 9.3) [36]. An analysis of identical samples confirmed that PSD with MALDI and DIOS produced frag-

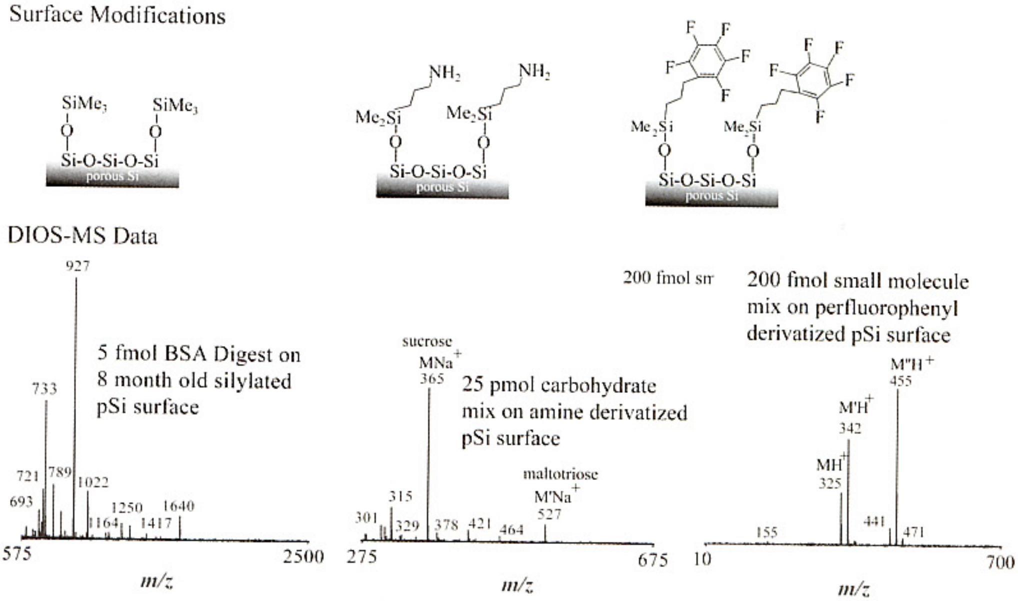


Fig. 9.2 By using different chemical functionalities, pSi surfaces can be tailored to selectively adsorb and efficiently ionize analytes. Left to right: DIOS-MS spectra of 500 fmol bovine serum albumin (BSA) digest analyzed on an 8-month-old TMS-derivatized DIOS chip, carbohydrate mix containing sucrose (MNa$^+$ 365) and maltotriose (M′Na$^+$ 527) on an amine silylated pSi surface, and small molecule mix containing midazolam (MH$^+$ 325), propafenone (M′H$^+$ 342), and verapamil (M″H$^+$ 455) on a perfluorophenyl silylated pSi surface. The hydrophobic TMS- and perfluorophenyl-derivatized surface is amenable to hydrophobic molecules, while the amine derivatized surface is more amenable to hydrophilic molecules.

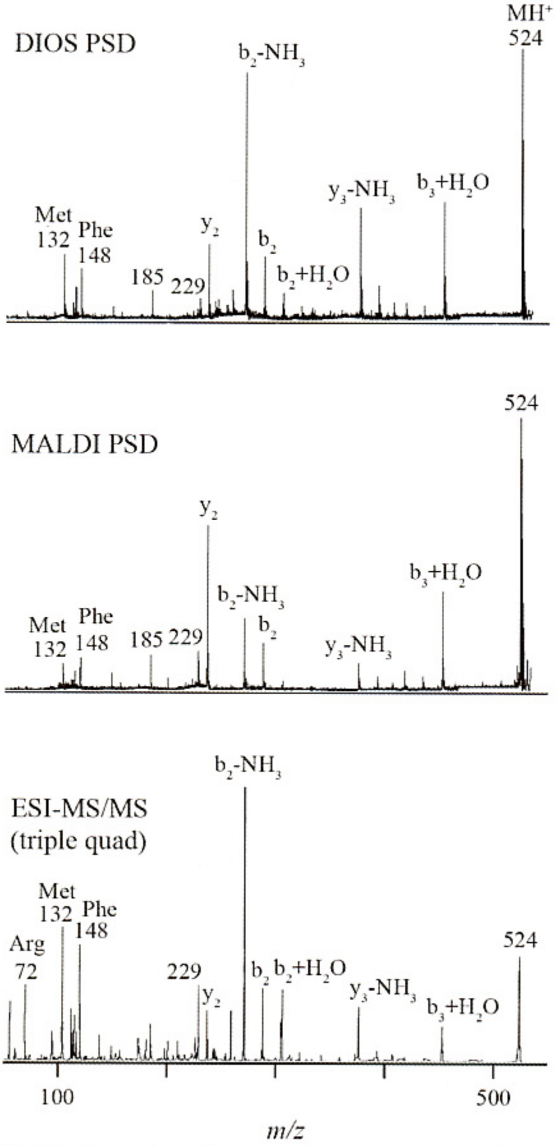


Fig. 9.3 Comparison of DIOS and MALDI post source decay (PSD) data, in addition to electrospray triple quadrupole tandem mass characterization of the peptide MRFA. In the characterization of smaller molecules ($m/z < 500$) by MALDI PSD (not shown), interference from background ions was observed in the fragmentation spectrum.

ments similar to those observed with ESI low-energy CID MS/MS, and can be used for structural elucidation.

In a very recent development, silicon nanowires (SiNWs) have been used as an alternative platform for DIOS-MS [38]. Unlike pSi surfaces, SiNWs are grown on the surface of a solid Si-substrate. In this study, SiNWs are prepared by vapor-liquid-solid (VLS) growth mechanism using Au nanoclusters as growth catalyst [39]. Size-selected Au colloid particles were deposited onto silicon wafers to produce high-quality SiNWs with a narrow diameter distribution. By tailoring the nanowire density, size, and growth orientation, the DIOS performance of SiNWs as a platform for matrix-free MS was examined using peptides and small drug molecules as model compounds. A recently developed chemical modification on ozone-oxidized pSi, which has proven essential to achieve high sensitivities, was employed on the SiNWs [37]. Remarkably, DIOS from SiNWs required much less laser fluence to desorb/ionize analytes compared to pSi and MALDI. As a result, very few surface-related background ions were observed from SiNWs. Although the detection limit obtained for small molecules was comparable with that for silylated pSi surfaces, the detection sensitivity of peptides was six orders of magnitude lower than for pSi. The detection can be further improved by optimizing the fabrication parameters and silylation chemistry. In addition to its ability to support laser desorption and ionization, SiNW has strong fluid wicking properties, these being driven by capillary action generated in the interstitial spaces between SiNWs. This property was exploited in the chromatographic separation and subsequent MS analysis of endogenous analytes from human serum and small drug molecules in biofluids (see Section 9.6).

The applicability of ordered nanocavity arrays as SALDI substrate in the analysis of peptides and plant metabolites has been reported recently [40]. These Si nanocavities are about 200 nm in diameter and 200 nm deep, and are fabricated by nanosphere lithography coupled with reactive ion etching. Desorption/ionization characteristics of the arrays were found to depend heavily on the surface morphology. An analysis of small peptides ($m/z < 500$) from this arrayed surface shows analyte peaks with S/N ratios comparable to those for pSi surfaces. However, for large peptides more background ions were observed due to the higher laser fluence needed for desorption. Plant metabolites from *Arabidopsis thaliana* root extract were successfully detected from this arrayed surface with minimal background ions.

9.3
Sample Preparation

Sample preparation plays a critical role in obtaining good-quality mass spectral data. Optimal MALDI sample preparation should provide good sensitivity as well as reproducibility. A number of sample preparation methods have been developed, depending on the type of analyte. Among the most commonly used MALDI sample preparation method (mainly due to its simplicity) is the dried droplet method; this entails mixing the analyte solution with the matrix material dissolved in an organic solvent/water mixture with an appropriate matrix:analyte ratio (≥10:1), deposition on the MALDI probe, and then air-drying. The evaporation of the solvent allows for co-crystallization of the analyte and matrix. This crystallization process usually results in the heterogeneous distribution of analyte within the sample spot, which in turn leads to poor shot-to-shot and sample-to-sample reproducibility. Several sample preparation methods are discussed in the following section to address this issue.

9.3.1
Electrospray Sample Deposition

Numerous developments in MALDI sample preparation methods have focused on improving sample homogeneity. Among these methods, electrospray sample deposition (ESD) is the most effective technique to achieve sample homogeneity. ESD, when coupled with MALDI-MS, has been reported to markedly improve sample homogeneity and result in enhanced sensitivity and signal reproducibility [41,42]. In ESD, the analyte solution is drawn into a syringe and fed to a needle mounted on an XY translation stage. To generate the electrospray, a voltage difference is applied between the needle and the MALDI target plate. Electrospraying results in the formation of a very fine mist of positively charged droplets that rapidly dry and are dispersed into a homogeneous circular pattern on the target plate. Spray stability is dependent on the flow rate, the shape and size of the capillary tip, the potential difference, the solvent system, and the distance between the needle and the target plate. Typical electrospray conditions include low flow rates (1–20 μL min^{-1}), an electrospray needle with inner diameter <10 μm, a potential difference of about 2–10 kV, analyte and matrix solutions in volatile solvent, and a separation distance of ~1 mm between the needle and the target plate. By optimizing these conditions, ESD yields a uniformly distributed analyte within the sample spot.

MALDI sample preparation for ES deposition involves mixing a saturated solution of the matrix with the analyte solution at a ratio of 1 : 1 (v/v) in a volatile solvent. Samples are introduced into the electrospray capillary and deposited onto a MALDI target. The spray conditions, including voltage, spray distance and flow rate, are optimized such that analyte particles arrive at the MALDI target plate with some solvent. It has been reported that dry deposition produces small or no crystal on the target plate, and this results in a weak MALDI signal [43]. In the same study, wet deposition was demonstrated to yield detection limits in the low attomole range for peptides.

9.3.2
Analyte Derivatization

Despite the successful application of MALDI and DIOS in the analysis of LMM compounds, the analysis remains a challenge. This stems primarily from low ionization efficiencies due to a lack of functional groups with high proton affinity, matrix suppression effects, isobaric overlay of the matrix with the analyte, and the high volatility of many analytes. One approach to improve the analysis of LMM compounds is by derivatization. Derivatization of samples prior to MS analysis has been widely employed in gas chromatography-mass spectrometry (GC-MS) [44–53] and ESI-MS [47,54–57], but very few MALDI [58–60] and DIOS [61] studies have been reported. The derivatization of LMM compounds prior to mass analysis offers the advantage of increased ionization efficiencies due to the incorporation of high proton affinity functional groups, differentiating matrix from analyte signals due to the increase in observable mass, stabilization of analytes, and facile introduction of isotopic labels for analyte quantitation. Several straightforward one-pot derivatization schemes have been reported in the analysis of biochemically relevant small organic molecules such as alcohols, carboxylic acid, keto-carboxylic acids, aldehydes, ketones, and amines [62]. This simple derivatization procedure allowed for the rapid and efficient analysis of a wide variety of small organic molecules, and provided clear distinction among isobaric compounds due to a specific increase in the mass of the analytes. The limit of detection for each class of organic compound was found to be similar for both derivatized and underivatized samples. Another derivatization procedure reported recently for small-molecule MALDI is the addition of a large, positively charged tag to the analyte using *N*-hydroxysuccinimide ester [63]. This derivatization reagent reacts specifically with LMM compounds with mass <500 Da containing either primary or secondary amine functional group, and results in a mass shift of 573 Da. The addition of a large positive tag to the analytes significantly improved sensitivity and the detection limit in the low femtomole range compared to underivatized samples, and also allowed for the analysis of samples without further purification. In addition, due to the ease of incorporating isotopically labeled tag, antibiotic mixtures were successfully quantified.

9.3.3
Analyte Pre-Concentration

9.3.3.1 Prestructured Sample Supports

One of the key steps in the dried droplet method is crystallization of the analyte-matrix mixture. During the crystallization process, a heterogeneous distribution of analyte containing crystals is formed over a spot size of ~5–15 mm^2. With only small portions of the dried sample irradiated by the laser, "sweet-spots" are observed which impart a limitation in the quantitative analysis of LMM and sample throughput. One way of solving this problem is to deposit the sample onto nitrocellulose substrates [64]. Indeed, Russell et al. have shown that the use of nitrocellulose as MALDI substrate resulted in an increased yield of protonated peptide signal and significant improvements in reproducibility and the precision of quantification. These authors suggested that nitrocellulose substrate modified the crystallization of the matrix-analyte solution and allowed more uniform coverage over the sample surface. Use of the nitrocellulose substrate produced a linear standard curve with a correlation coefficient of 0.991 in the range of 4 to 200 pmol during the analysis of bradykinin.

An alternative approach is to reduce the sample spot size close to the diameter of the laser spot. One way of doing this is to apply a small volume of the sample solution (typically a few nanoliters) onto a MALDI target plate. This sample preparation technique requires state-of-the-art liquid handling systems, with the ability to pipette volumes in the low nanoliter range to enhance sample utilization by pre-concentration. Sample pre-concentration provides the practical benefits of improved sensitivity, better shot-to-shot reproducibility, and less signal suppression.

Another sample pre-concentration technique that can be utilized in the analysis of LMM compounds is the use of MALDI Anchor-Chip™ (Bruker Biosciences) [65,66]. This sample support system consists of an array of small gold hydrophilic center (diameters from 200 to 800 μM) surrounded by a Teflon-coated hydrophobic exterior region. Aqueous samples deposited are exclusively held in place in the hydrophilic center due to the surrounding Teflon material, which repels the sample droplet. Samples are enriched before matrix crystallization, and this results in an improved detection sensitivity, provided that the also increased concentration of impurities does not interfere with the MALDI process. For example, MALDI analysis of peptides using Anchor-Chip™ showed detection limits in the low attomole range.

An alternative, less-expensive method to produce disposable sample supports has been described by Owen et al. [67], in which blank stainless-steel plates were coated with Teflon and Scotch Guard™ hydrophobic coatings. Enhancement in detection sensitivity correlated with reduced spot sizes when using the hydrophobic coatings, which could be removed by cleaning and reapplied between uses [67].

9.3.3.2 DIOS with Solid Liquid Extraction

The ease of incorporating functional groups to modify the pSi surface for mass spectrometric application has allowed for the development of a convenient and simple method for enriching the concentration of analytes. This technique, termed DIOS solid-liquid extraction (SLE), uses the property of differential adsorption selectively to capture analytes from a solution containing contaminants that impede MS analysis. Hydrophobically modified DIOS surfaces could be readily used selectively to remove interferences prior to analysis. DIOS SLE simply involves the deposition of a droplet containing analytes onto the chemically modified DIOS surface; then, after approximately 3 s, the sample is aspirated with the same pipette (Fig. 9.4). This short-term deposition allows for any molecule with a propensity for adsorption onto the surface to attach itself, yet any potential hydrophilic contaminants such as salts and buffers remain in solution, leaving the surface free of such contaminants. The van der Waals interaction between the analyte and surface selectively extract the small molecules, while the hydrophilic contaminants such as salts are removed with the droplet. DIOS analysis of peptides and small molecules from complex matrices are greatly enhanced by differential adsorption [37].

The applicability of DIOS SLE is demonstrated in the DIOS analysis of endogenous analytes in blood plasma. Using a simple cold methanol extraction to remove the proteins followed by a butyl ester derivatization, amino acids such as phenylalanine, alanine, isoleucine/leucine, glutamic acid, and the doped deuterated internal standard of phenylalanine, used to quantify the amino acid,

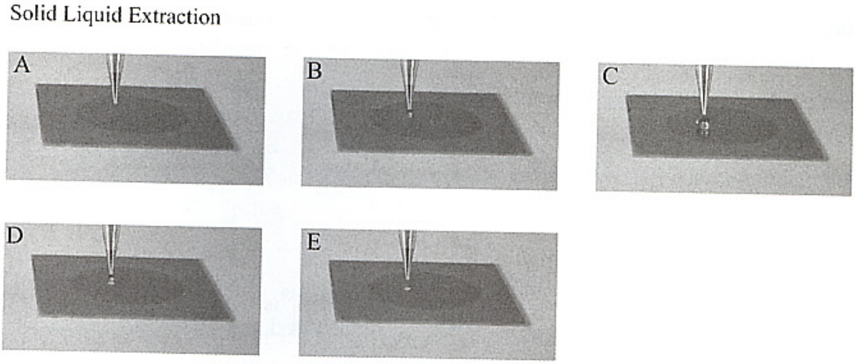


Fig. 9.4 Analyte clean up with DIOS solid-liquid extraction (SLE). This simply involves the deposition of a droplet containing analytes such as a protein digest and its subsequent removal with a pipettor. The Van der Waals forces between the analyte and surface selectively extract the peptides or small molecules, while the hydrophilic contaminants such as salts are removed with the droplet.

were detected with good sensitivity (Fig. 9.5). Using DIOS PSD analysis, the detection of the amino acid in blood plasma was confirmed by monitoring the MS/MS fragmentation pattern of the butyl ester derivative of phenylalanine and its deuterated analog. DIOS PSD analysis shows a characteristic neutral loss of 102 Da associated with butyl formate resulting in the product ions at 120 and 125 Da from the collision-induced dissociation (CID) of butylated phenylalanine and its deuterated internal standard, respectively (Fig. 9.5).

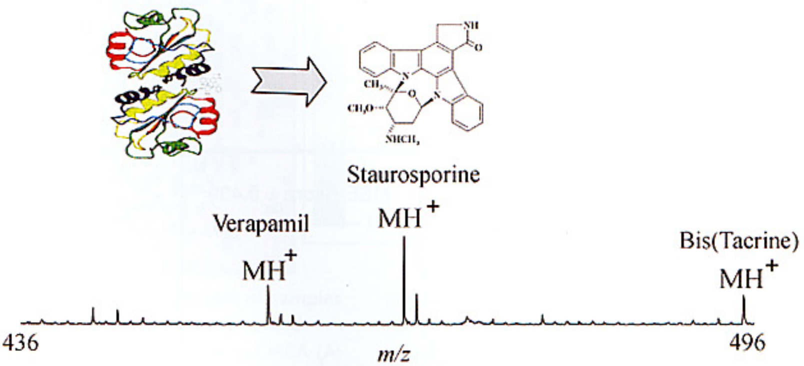
The value of DIOS SLE on silylated pSi surfaces was further demonstrated in the analysis of drug molecules from drug–protein complexes. The presence of high quantities of salt and protein often results in the formation of a crust on the target, which inhibits both MALDI and DIOS-MS analyses. In addition, the dried droplet method containing the denatured protein always fails to release the drug molecule in both DIOS and MALDI. By employing DIOS SLE, most contaminants such as polar salts can be removed by differentially adsorbing the analytes onto the silylated pSi surface. The selective extraction of the potent kinase inhibitor, staurosporine, in the presence of two other unbound molecules by using a 10 kDa MW cut-off filter to isolate the Rho-kinase II–staurosporine complex, spotting the sample onto the DIOS target, and then removing polar interferences prior to MS analysis is shown in Figure 9.6. The hydrophobic interaction of the drug with the perfluoroalkyl-modified DIOS surface allowed the drug to be extracted, even in the presence of protein, salts and stabilizing agents for the active enzyme (25% glycerol, 75 mM NaCl, 0.5 mM benzamidine, 0.1 mM PMSF, 0.05 mM EGTA, 0.015% Brij 35, 0.05% 1-mercaptoethanol in the original mixture). Thus, SLE serves as a useful tool for monitoring drugs in biological fluids in the presence of large amounts of proteins and soluble non-volatile contaminants.

Fig. 9.5 (A) Solid-liquid extraction (SLE) followed by DIOS mass analysis of amino acids in human plasma after esterification. (B) DIOS PSD profile of phenylalanine ($M'H^+$ 222) and d_5-phenylalanine (MH^+ 227) showing a characteristic neutral loss of 102 Da corresponding to butyl formate. This results in the product ions of the butyl ester derivatives of phenylalanine (MH^+ 120) and its corresponding deuterated internal standard ($M'H^+$ 125). Reprinted with permission from Ref. [37]; © American Chemical Society, 2004.

9.3.4
Matrix Suppression

Sample preparation can be tailored to suppress excess matrix background peaks by controlling the matrix : analyte ratio. Although numerous groups observed this phenomenon soon after the introduction of MALDI, it has only recently been exploited to achieve its full potential for small-molecule analysis. The matrix : analyte molar ratio within the matrix crystals has been found to be critical for MALDI quantification [10]. Critical parameters for successful execution of the matrix suppression effect (MSE) include sufficient analyte and optimized laser intensity. As in the case of FAB-MS, the use of surfactants such as cetrimonium bromide (CTAB) can be used to substantially or even completely suppress the matrix-

(a) DIOS-MS on a Mixture of Rho-kinase II and Potential Inhibitors



(b) DIOS-MS on the Mixture After 10 kDa MW Cutoff Filter

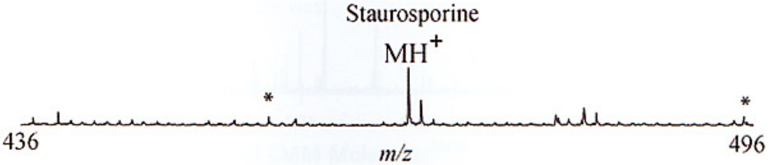


Fig. 9.6 Monitoring of enzyme inhibitors using SLE with DIOS-MS. Solid-liquid extraction (SLE) of a mixture of Rho-kinase II, staurosporine (a potent kinase inhibitor), verapamil and bis(tacrine) before (A) and after (B) passage through a 10 kDa MW cut-off filter in the presence of a stabilizing buffer allowed for the identification of a known inhibitor. The asterisks mark the position of verapamil (*m/z* 455) and bis(tacrine) (*m/z* 496) after depletion. Reprinted with permission from Ref. [37]; © American Chemical Society, 2004.

related ion background [68]. The use of CTAB surfactant also resulted in improved mass resolution for low-molecular weight molecules including amino acids, peptides, drugs, and cyclodextrins.

Knochenmuss and coworkers have extensively studied the MSE [69,70], and have introduced a system to calculate the extent of matrix suppression and its effect on qualitative spectral interpretation for LMM analytes. Figure 9.7 shows three MALDI-TOF mass spectra of caffeine, each exhibiting different MSE scores. These scores are calculated using the equation:

$$\text{MSE score} = \frac{\sum A}{\sum A + \sum M},$$

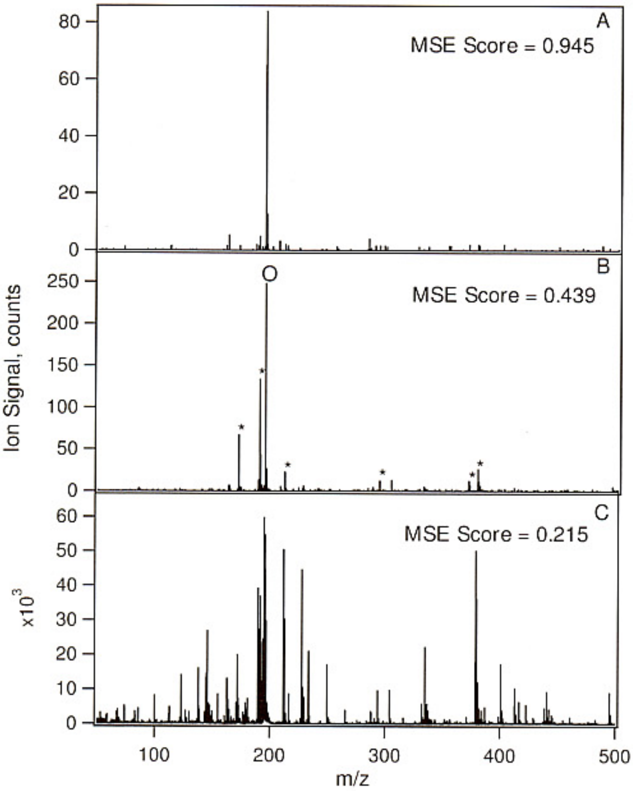


Fig. 9.7 Three MALDI-TOF mass spectra of caffeine, including the matrix suppression effect (MSE) scores. The matrix used was α-cyano-4-hydroxycinnamic acid (α-CHCA). Peaks marked with asterisks in (B) indicate matrix signals included in the calculation of the MSE score, while the signal from the protonated analyte is marked with "O". MSE is nearly complete in (A). In (C), many more matrix signals appear than in (B). These were not included in calculating the score, which would otherwise be even lower. The matrix: analyte mole ratio was 3 in spectra (A) and (C), and 27 in spectrum (B). Low laser intensity was applied for measurements in (A) and (B), and a high laser intensity was used for spectrum (C). Reprinted with permission from Ref. [71]; © American Chemical Society, 2004.

where M is the sum intensity of predefined matrix peaks and A is the intensity of the predefined analyte peak. MALDI images can be generated and filtered using MSE scores to monitor analyte homogeneity across sample spots and identify areas where optimal signals can be generated. Filtered MALDI images of a mixture of yohimbine and caffeine are shown in Figure 9.8. The green pixels represent areas where the correct ratio of yohimbine to caffeine was determined. Isolated areas of elevated caffeine signal can be identified indicating local fractionation during preparation, particularly for samples prepared with DHB [71].

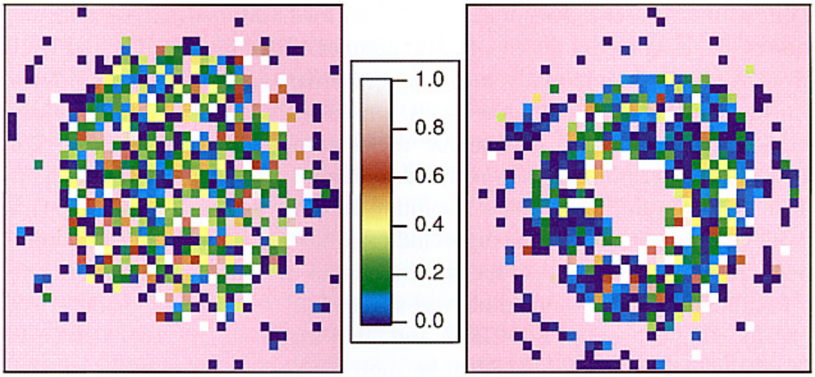


Fig. 9.8 Filtered MALDI images of samples containing a mixture of yohimbine and caffeine analytes with matrixes α-CHCA (A) and DHB (B). The ratio of yohimbine to caffeine to matrix was 4 : 1 : 36. In the absence of analyte suppression effects, the ratio of caffeine to yohimbine signals is expected to be 0.25. The yohimbine MSE score was used as a filter. The ratio of caffeine to yohimbine was calculated only if the MSE score was greater than 0.9; otherwise, the pixel was left blank (pink areas). The expected ratio was 0.25 (green pixels). Local regions of unexpectedly low (blue pixels) and high caffeine signal (white pixels) are observed. This may be an indication of local fractionation during sample drying. Reprinted with permission from Ref. [71]; © American Chemical Society, 2004.

9.4
Qualitative Characterization of LMM Molecules

Arguably, small-peptide analysis is one of the largest areas of MALDI application to LMM compounds. Chen et al. [72] initially demonstrated the applicability of MALDI to study small peptides (800–1500 Da). A variety of excellent examples of MALDI for the analysis of small peptides can be found in almost every MALDI publication [73], and it is probably safe to say that MALDI has become the method of choice for peptide analysis. Furthermore, some small peptides such as angiotensin and substance P have become "gold standards" for the verification of matrix and instrument performance [4]. The analysis of peptide maps for the characterization of proteins is discussed in detail in Chapter 3.

One other well-established area of MALDI is that of carbohydrate analysis. It has been shown that MALDI is applicable for the analysis of underivatized and derivatized carbohydrates, and can also provide complementary data to other techniques [74–77]. MALDI matrices and sample preparation techniques used in carbohydrates analysis have been reviewed in detail by Harvey [76]. Interestingly, carbohydrates themselves have been used to improve MALDI signal reproducibility and mass resolution of other analytes via improved matrix crystal homogeneity and potentially gas-phase cooling of analyte molecules [78–80]. Further details relating to the MALDI-MS of carbohydrates and glycoconjugates can be found in Chapter 6.

In one of the earliest reports utilizing MALDI for LMM analytes, Duncan and coworkers [81] examined a variety of LMM compounds using α-CHCA and DHB matrices. A range of compounds, including quaternary ammonium salts, sterols, nucleosides, purine and pyrimidine bases, amino acids, catecholamines, opiods, antibiotics, prostaglandins, macrocyclic metal complexes of porphyrins, and phthalocyanines, was successfully analyzed. Although these authors utilized a linear TOF system with limited mass resolution and conventional sample preparation with crystalline matrices, the report indicated that the majority of compounds studied gave interpretable mass spectra. In another application of MALDI, a series of tetrathiofulvalene compounds was successfully studied and characterized [82], again using conventional α-CHCA and DHB matrices. The results obtained for 26 compounds showed MALDI to be more viable than other mass spectrometric methods for a broad spectrum of chemical materials.

MALDI methodology has been successfully applied to both polar and non-polar retinoids [83]. Although MALDI spectra exhibited background ions from the DHB matrix, the analyte ions were easily identified. Furthermore, the specific structural fragments observed provided additional data to assist in retinoid characterization. Interestingly, only 4-oxoretinoic acid was detected as a protonated species (versus radical molecular ions), even under LDI conditions. The authors suggested that this might be the result of a higher proton affinity of the oxo-retinoic acid compared to the other retinoic acids.

UV MALDI has also been used to characterize complex mixtures of hazardous waste [13]. Three classes of compounds were studied, namely organic acids, salts of oxyanions, and amine-based chelators. The organic acids exhibited strong signals in negative-ion mode, whereas the chelating compounds showed strong signals in positive-ion mode. It was also found that a high sodium concentration could quench MALDI signals. The implementation of an off-line clean up with ion-exchange column prior to MALDI analysis yielded a 100-fold sensitivity increase.

The early applications of MALDI for pharmaceutical compounds have focused primarily on the characterization of compounds not amenable to ESI-MS determinations. MALDI has been utilized for simple identification and accurate mass determination of the anti-hypertensive prazosine and its synthetic analogues [84], the anti-malarial double deuterium-labeled ferrochloroquine [85], and a glucuronide metabolite of an H_1 antihistamine, dimethindene [86].

Metabolism studies, though not numerous, have highlighted the problem-solving ability of MALDI. In one study, Morvan et al. monitored the fate of an oligonucleotide dodecathymidine prodrug in cell extracts, whereby the kinetics and metabolic half-life of the prodrug were determined, and the prodrug was fully metabolized to T_{12} phosphorothioate [87]. An unknown metabolite of the antiviral acyclic nucleotide analogue cidofovir was isolated from rat kidney and confirmed by MALDI and NMR to be cidofovir-phosphocholine [88]. Olson and Fabris have shown that MALDI can also be used as a tool to determine metabolites *in vitro*, via CYP_{450} enzymes in liver microsomes, of tamoxifen, promethazine, and diphenhydramine [89].

Siuzdak and coworkers have examined the intracellular transport of cationic drugs (notably the tetraphenylphosphonium cation) by using MALDI-MS. The cation was quantified at subpicomole levels in cell lysates, and the biodistribution-based drug resistance characterized. The results obtained by MALDI were comparable to those produced by scintillation counting [90]. The use of MALDI PSD and CID analyses and MALDI PSD/CID has been compared to ESI trap characterization for 4-quinolone antibiotics and oleandomycin. The choice of matrix was found to control the amount of fragmentation, with the TiO_2 matrix exhibiting less background interference but also fewer fragments for identification [91]. Nicotine- and cotinine- (a nicotine metabolite) adducts of melanin have been studied as a model to investigate the incorporation of drugs into hair. Adducts of monomeric melanin dopaquinone (DOPAQ) with nicotine and cotinine were determined and compared to DOPAQ adducts with deuterated analogues of nicotine and cotinine [92]. Not surprisingly, one logical pharmaceutical application of MALDI has been in the realm of phototherapy, though few systems have been investigated; photofrin, a porphyrin derivative used to treat tumors [93], temoporfin, a tumor-localizing photosensitizer [94], and a dye-dendrimer complex, pheophorbide-a-substituted diaminobutane polypropyleneimine [95].

MALDI may also be a method of choice for the rapid and automated analysis of combinatorial libraries in the presence of buffers and contamination [96–98]. One appealing aspect of the MALDI method is that the UV laser light can be used simultaneously to promote photolytic cleavage of the analyte from the solid support and its gas phase ionization for subsequent mass spectral analysis. The general utility of termination synthesis with MALDI analytical methods has been illustrated with a non-linear, non-peptide cyclic oligocarbamate library [99]. Siuzdak and Lewis have shown that peptides and carbohydrates, covalently linked to a polymeric support through a photolabile linker, can be directly identified by MALDI in a single step which requires no pretreatment of the sample to induce cleavage from the support [100]. In another application [101], MALDI was used for the photolytic release and determination of an active glycopeptide from the resin support. The resulting mass spectral data contained the ladder of glycopeptide fragments and yielded the active glycopeptide sequence. A small library of chymostatin derivatives has been synthesized, with 22 compounds identified by MALDI [102]. The "one-bead–one-compound" (OBOC) combinatorial method has also been successfully supported by MALDI analysis using an isotopically tagged encoding strategy to drive unambiguous identification based on bromine and chlorine patterns within the data generated [103,104]. A robust, high-throughput method was also validated for a model 12288-member library that was screened against streptavidin [104].

In general, surfactants are confounding mixture components which interfere with biomolecule characterization by MS, and sample clean-up steps are necessary prior to analysis. MALDI analysis of LMM ionic and nonionic surfactants has been conducted by a number of groups. Several different detergents including Triton X-100 and 114, Tween 20 and Brij 35 were analyzed by TLC, reverse-phase HPLC and MALDI with comparable results being obtained among the techniques

[105]. In the same studies, Mega 8 *n*-octylglycoside, Chaps, Chapso, sulfobetaine, and the zwitterionic surfactant SB14 were examined, with additional methylene group-containing impurities being detected in some samples. Attempts to remove matrix interference by addition of silver salt were unsuccessful.

Ayorinde and coworkers have analyzed several types of surfactants, including nonionic polysorbates Tween 20, 40, 60, 80, all of which are fatty acid esters of polyethoxysorbitan. Complex mixtures of PEGs, PEG esters, and isosorbide polyetholuxlate were detected. During the analysis, seven different series of oligomers ions were present due to mixture of fatty acid esters [106]. Nonylphenylethoxlate surfactants (Surfonic®) which can serve as a carbon source/substrate to produce polyhydroxybutyrate for bacterial degradation have been investigated by this group [107]. Either a higher molecular weight (non-interfering) porphyrin matrix or α-CHCA were utilized as matrices for the Surfonic samples, although higher sample concentrations were necessary for the α-CHCA matrix. Monomer and dimer peaks were not observed in the MALDI spectra of these samples. Other classes of surfactants analyzed by MALDI include Surfynol [108] and quaternary ammonium compounds [109].

9.5
Analyte Quantitation by MALDI

The use of MALDI technique in quantitative analysis of LMM compounds has been well documented and reviewed [110]. However, despite numerous reports [11,73,111–115], analyte quantitation by MALDI is still considered to be a highly unreliable procedure. Some of the critical issues associated with quantitative MALDI-MS include: (i) instrumentation, related mass resolution and accuracy; (ii) linearity and precision of the data acquisition system; and (iii) sample preparation and signal reproducibility. Mass resolution and linearity of acquisition systems had been one of the major obstacles in the early applications of MALDI [116]. However, technological advances in MS instrumentation – including developments in delayed extraction [6,7,117], powerful ion detectors, high-repetition lasers, AP-MALDI interfaces, and fast speed acquisition systems – have adequately addressed most of the instrumental issues. The main limiting factor in quantitative MALDI is sample preparation, and associated with this are *intra* (sample-to-sample) and *inter* (point-to-point and shot-to-shot signal) experimental reproducibility. The best way to compensate for signal deviation in quantitative MALDI is to use an internal standard (IS); some examples summarizing the major approaches for sample preparation and selection and utilization of IS for quantitative characterization in the LMM range are provided in the following sections.

9.5.1
Selection of IS

The correct selection of an IS is critical in achieving accurate analyte quantitation and acceptable standard curve linearity of biologically interesting LMM compounds. The use of an IS can minimize variability in analyte signal intensities and improve experimental reproducibility [113]. One requirement needed for an IS is that its physico-chemical characteristics should be identical to those of the analyte of interest during the measurement, in order to ensure similar ionization behavior. In addition, the mass separation between the analyte and IS must not overlap for a straightforward measurement of peak intensity. Traditionally, isotopically labeled compounds are used as internal standards, but in some cases when these are unavailable then structural homologues or analogues have been used as an alternative.

The quantitative characterization of LMM biomolecules (<500 Da) has been demonstrated by Duncan and coworkers [81]. Different types of IS for the stable isotope-labeled analyte (^{13}C and deuterated) and structural analogues were evaluated. Three different systems were examined: (i) 3,4-dihydroxyphenylalanine (DOPA) for the ^{13}C-labeled system; (ii) acetocholine for deuterated analogues as IS; and (iii) two synthetic peptides, differing only slightly in amino acid composition. The acetocholine system was shown to provide the best linearity, with a correlation coefficient of 0.98 for the standard curve. The analyte and IS peak were well-resolved, and no interference occurred with the DHB matrix peaks. These authors also noted that use of the ^{13}C label on six carbons compromised the results for DOPA system due to poor mass resolution. Thus, although a linear standard curve was obtained (correlation coefficient 0.967) for the ^{13}C-DOPA system, resolution of the IS from the analyte was crucial for quantitative characterization.

9.5.2
Methods for Improving Quantitative Performance

An instrumental protocol for MALDI data collection can also be a powerful tool for minimizing signal deviations [18,69,71,118]. Data acquisition at constant laser energy was compared to data acquisition with constant ion abundance [118]. The latter studies showed improved reproducibility, as well as allowing more consecutive laser shots at the same position. A linear standard curve (r = 0.9997 from 0.05 to 30 ng mL^{-1}) was demonstrated for chlormequat using an isotopically labeled IS and data collection using constant ion abundance.

The dependence of the RSD of the analyte : IS ratio on the number of laser shots has been studied by Gusev et al. [18,119]. These authors found that the maximum signal intensity was obtained for the first 40 laser shots, and the lowest RSD was achieved for the 40 laser shots after the first 10. A higher percentage RSD for the first 10 shots was explained by the presence of contamination at the surface, which causes deviations in the analyte : IS ratio. In order to avoid these deviations, the authors suggested discarding the first 10 shots, which halved the RSD of analyte : IS ratio. A similar protocol was shown to be effective for fast evaporation [120] and dried droplet sample preparations [121]. An instrumental data collection protocol can also be automated using on-line correlation analysis in order simultaneously to improve mass resolution and to minimize signal deviations [120].

9.5.3
Quantitation of Pharmaceutical Compounds

In recent years, MALDI quantitation has shown the most progress in the pharmaceutical arena, due mainly to the ever-present demand for higher throughput analytical methods. On the basis of the availability of high-repetition lasers and AP MALDI sources, reports continue to emerge in this area. For example, LeRiche et al. showed that, for 4-quinolone antibiotics and oleandomycin, MALDI with PSD and PSD/CID generated a higher number of different product ions than ESI trap MS^n (with $n \leq$) [91]. In a recent report, AP-DIOS was utilized in the forensic analysis of tablets seized during drug raids [122].

The use of high-repetition lasers has facilitated its coupling to analyzers other than TOFs, and has also driven real-time analysis of three or more samples per minute, which is the current benchmark for reasonable throughput analysis by ESI-LC/MS/MS. Hatsis et al. have demonstrated successful quantitation of benzodiazepines using either quadrupole-TOF or triple quadrupole mass spectrometers with a frequency-tripled (355 nm) Nd:YAG laser operating at 1 kHz [9]. Sleno and Volmer have optimized various instrumentation and sample preparation parameters for LMM quantitation; here, α-CHCA was found to be a better matrix compared to DHB or sinapinic acid for the compounds examined, the data correlating with reports from other groups [123,124]. Sample consumption was in the 1 femtomole range, offering significant advantage over ESI LC/MS/MS.

Gobey et al. have reported an exhaustive comparison of ESI and MALDI quantitation as applied to high-throughput *in-vitro* screens [124]. In this study, 53 diverse compounds were assayed through a metabolic stability screen using human liver microsomes and split for MALDI and ESI-LC/MS/MS analyses. A typical data collection profile is shown in Figure 9.9. In this experiment, test compounds were subjected to incubation with human liver microsomes, an *in-vitro* model that simulated how analytes are metabolized in humans. If the test compound is liable to undergo biotransformation via the enzymes found in human liver microsomes, then the amount of test compound remaining over the course of the experiment represents how susceptible the compound is to metabolism. Thus, plotting the amount of analyte remaining as a function of time as monitored by either analyte peak area or analyte:IS peak area ratio allows an assessment to be made of analyte metabolic stability and half-life.

Samples were prepared by first terminating the incubation reaction by the addition of acetonitrile, followed by SPE using an Oasis HLB 96-well extraction procedure. Both the α-CHCA matrix and IS were added as part of the SPE elution solvent mixture. After extraction, samples were spotted onto the target plate, with contiguous standards and aliquots from each incubation time point. The entire sample set was then rastered past the laser beam and collected into a single chromatogram (see Fig. 9.9). Half-lives were calculated using the more accurate and reproducible analyte:IS peak area ratios. A comparison of half-lives determined by MALDI and ESI is shown in Figure 9.10. In general, good agreement was seen between the two techniques. MALDI quantitation was also applied to other

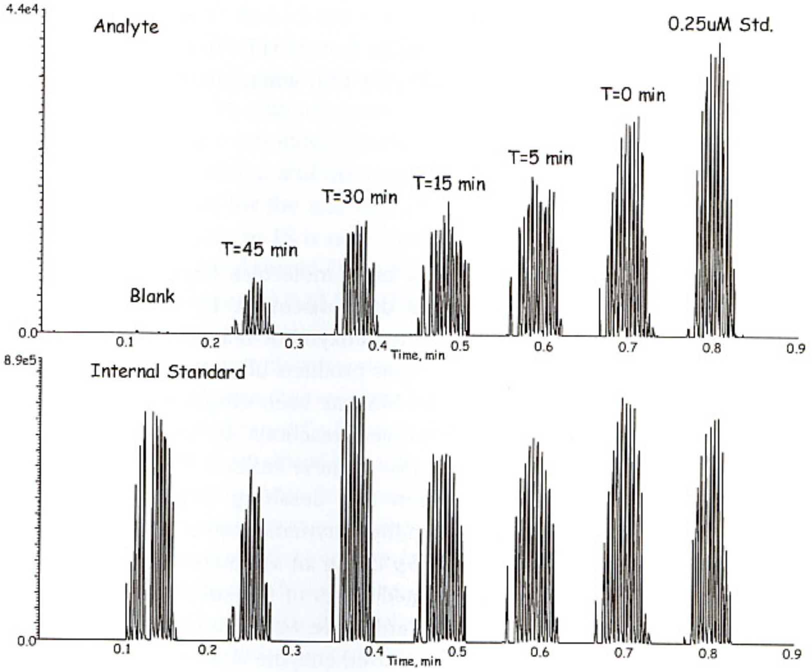


Fig. 9.9 Example of microsome incubation time course. Reprinted with permission from Ref. [124]; © American Chemical Society, 2005.

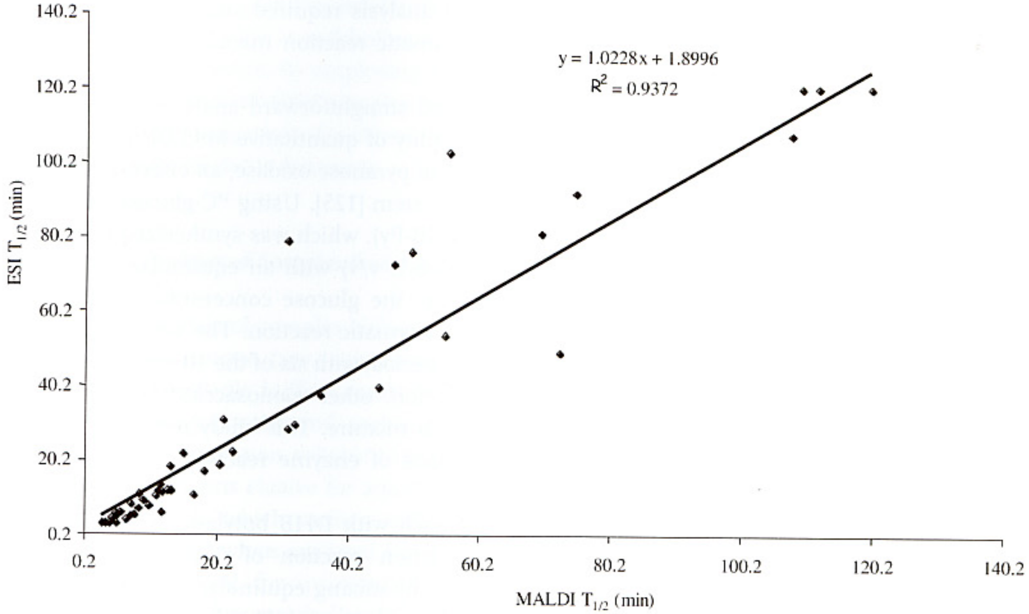


Fig. 9.10 Human microsome half-lives for 53 compounds: ESI versus MALDI. Reprinted with permission from Ref. [124]; © American Chemical Society, 2005.

in-vitro assays such as human hepatocytes, Caco-2, and MDR Pgp transport. However, ESI demonstrated better universality than MALDI in ionization success (15.9% failure rate for MALDI versus 6.7% for ESI) when analyzing a set of 208 compounds [124].

9.5.4
Enzyme Activity and Inhibition Studies

The development of efficient methods to assay molecules for catalytic enzyme activity is of considerable importance in drug discovery. In this respect, MS has emerged as a versatile tool in monitoring enzymatic reactions because it can directly detect most natural substrates and/or products of enzymatic reactions in a time-resolved fashion. To date, MALDI-MS has been employed in a number of quantitative analyses of enzyme-catalyzed reactions by monitoring LMM substrates and products [10,71,125]. In most of these studies, MALDI analysis is performed without additional purification and desalting steps. For example, quantitative MALDI has been employed in the enzymatic conversion of glucose to gluconolactone using DHB as matrix [125]. Using an optimized sample preparation method and ^{13}C-glucose as IS, sodiated peaks of the substrate and product were used for the analysis. A linear standard curve was obtained with an %RSD value of 6% out of 40 measurements. Moreover, enzyme activity was determined by monitoring the time course of disappearance of the substrate and appearance of the products, and compared with HPLC for method validation. Results from the time-course experiments obtained from MALDI and HPLC were in good agreement. However, unlike HPLC, MALDI analysis required small sample amount and allowed the direct analysis of enzymatic reaction mixture, without further purification.

In taking advantage of the simple and straightforward analysis of enzymatic reactions, these authors extended the utility of quantitative MALDI in screening enzyme activity using different variants of pyranose oxidase, an enzyme that converts glucose to glucosone, as a model system [125]. Using ^{13}C-glucose as IS and a liquid ionic matrix, DHB-pyridine (DHB-Py), which was synthesized by mixing DHB in acetonitrile/water/TFA (50:50:0.1, v/v) with an equimolar amount of pyridine for MALDI sample preparation, the glucose concentration was determined from samples obtained via the enzymatic reaction. The activity of 10 enzyme variants was monitored over a 1-h period, with six of the 10 enzyme variants used showing enzyme activity. In addition, other monosaccharides and sugar alcohols were detected from the reaction mixture. This study demonstrates the direct analysis of substrates and products of enzyme reactions in liquid ionic matrix, without a prepurification step.

The same MALDI-based assay approach with DHB butylamine (DHBB) was implemented to monitor the desialylation reaction of 3′-sialyllactose [126]. DHBB is a liquid ionic matrix prepared by mixing equimolar amounts of DHB and butylamine, and can be used in lieu of an organic solvent in the enzyme reaction, thereby allowing rapid monitoring of the enzyme-catalyzed reaction.

In another study, MALDI was employed in the quantitative analysis of LMM species generated from enzyme-catalyzed reactions using substrates as IS [10]. Because the substrate is consumed during the reaction, the measured signal ratios of the product and substrate are amplified, thereby reducing the experimental errors on the estimated kinetics. By optimizing the pH, matrix:analyte ratio, solvent composition, and the target plate, a good linear response ($r^2 = 0.985$, 0.999) was obtained for the analysis. This MALDI approach is potentially useful in enzyme screening, as IS is not required.

Recently, the applicability of DIOS-MS to monitor enzyme activity and inhibition studies has been reported [127,128]. The viability of DIOS-MS to monitor enzyme activity was demonstrated in the reaction time course of α-(2,6)-sialyltransferase in the sialylation of *N*-acetyllactosamine (LacNAc). Sialyltransferase is an important class of glycotransferase that exhibits high acceptor-substrate specificity, and also plays an important role in cell adhesion and molecular recognition events. By optimizing the reaction conditions, the time-course production of α-(2,6)-sialylated trisaccharide (m/z 475) was monitored from a single-step reaction catalyzed by α-(2,6)-sialyltransferase from a corresponding lactoside (m/z 788) in a period of 15 min. DIOS was also effective in monitoring the inhibition of acetylcholine esterase (AChE), a biologically relevant target enzyme used for enzyme screening inhibition studies. The pseudo first-order reaction of the hydrolytic catalysis of the neurotransmitter acetylcholine (m/z 146) to choline (m/z 104) was measured quantitatively using d_9-choline as internal standard and electrospray sample deposition. Once the kinetics was obtained, screening for potential AChE inhibitors was examined by monitoring the AChE activity in the presence of potential inhibitors from a small-molecule library. Known inhibitors were identified from the library through the observation of high acetylcholine:choline ratios; no false positives or false negatives were observed. By employing a standard commercial instrument with a 200 Hz laser, a total of 4000 samples was analyzed during the course of a 4-h period. This screening method is inherently sensitive, simple and quick, and should greatly facilitate inhibitor discovery.

9.5.5
Quantitative Analysis of Samples from Complex Biological Matrices

The characterization of complex biological samples presents a major challenge for any analytical technique. Low analyte concentrations, co-extracted endogenous materials, and nonvolatile buffers result in signal interference, signal suppression, and complicate qualitative and quantitative characterization. Despite significant strides in instrumentation, reliable MALDI quantitation without extensive sample pretreatment remains elusive for analytes in complex biological samples such as blood. Compounds, which perform well in neat solution, may exhibit significant losses in sensitivity when analyzed from blood or plasma. Even so, a handful of reports has emerged for quantitative MALDI analysis of amperozide [112], tacrolimus [113] and its metabolites [129], and cyclosporine A [130] and its metabolites [116] in plasma or blood. In general, MALDI results showed good correlation with results from other techniques such as ESI LC-MS/MS and HPLC/UV. Angiotensin II has been successfully quantitated in renal microdialysate fluid, a somewhat cleaner matrix than blood [80]. Desiderio et al. [111] have demonstrated the quantitative analysis of opioid peptide with pentadeuterated IS in ovine plasma samples. Based on the data obtained, the time profile in sheep plasma and the corresponding pharmacokinetic data were calculated.

Sample clean-up requirements still remain strict for MALDI analysis, with even low protein/lipid/carbohydrate matrices such as human urine demanding a two-step liquid/liquid extraction protocols [36]. However, recent reports of MALDI analysis using CNTs as matrix have shown the potential to circumvent multi-step sample clean-up, due to the sample extraction properties of the CNT matrix [131,132]. Pan et al. have demonstrated the analysis of a mixture of propranolol, quinine, and cinchonine in urine by mixing the analyte-containing urine sample with the CNT matrix, glycerol, and sucrose for maximum dispersion and adsorption of the analytes onto the CNT matrix [132]. Oxidized CNTs have been utilized in the analysis of three bioactive alkaloids, berberine, jatrorrhizine and palmatine, in extracts of traditional Chinese medicines using the method of standard additions [131].

9.5.6
Environmental Applications of Quantitative MALDI

The initial application of quantitative MALDI to environmental samples has focused on surfactants. Willetts et al. have studied the quantitation of Synperonic NP9, a potential contaminant in surface water [133]. The quantification of nonphenylethoxylates (NPEs) carries significant environmental importance due to their biodegradation to nonylphenol, which is known to cause an estrogenic response in breast cancer cells. LiCl was added to the sample preparation to simplify the observed oligomer distribution, and a quantification range of 10–50 $mg\,L^{-1}$ was obtained. Due to problems quantifying across the molecular weight distribution of the oligomers, separate calibration curves were obtained for each individual peak. The limit of detection was 80 $\mu g\,L^{-1}$ (compared to 10 $\mu g\,L^{-1}$ by HPLC). No Synperonic NP9 was detected in an actual water sample, but spiked samples were appropriately quantified by MALDI [133]. Ayorinde and Elhila have found that instrumental detection limits for NPEs are directly dependent on molecular weight. Low-molecular weight NPEs were detected at 10 $\mu g\,L^{-1}$, while higher-molecular weight NPEs showed detection limits as high as 4.5 $mg\,L^{-1}$ [134].

As the agricultural use of antibacterial drugs continues to rise, concerns have arisen regarding the accumulation of antibiotics in the soil and ground water, leading in time to increased drug resistance in human and veterinary medicine [135,136]. MALDI quantitation combined with Solid Phase ImmunoExtraction (SPIE) has been used for the analysis of sulfamethazine and its major metabolite, N^4-acetylsulfamethazine. The SPIE-MALDI procedure allowed rapid purification and detection of low ppb levels in water, soil, and manure samples [137].

Sleno and Volmer have utilized the enhanced capabilities of a MALDI-triple quadrupole mass spectrometer with a high-repetition laser to monitor spirolide toxins in phytoplankton [138]. A comprehensive analytical study was successfully

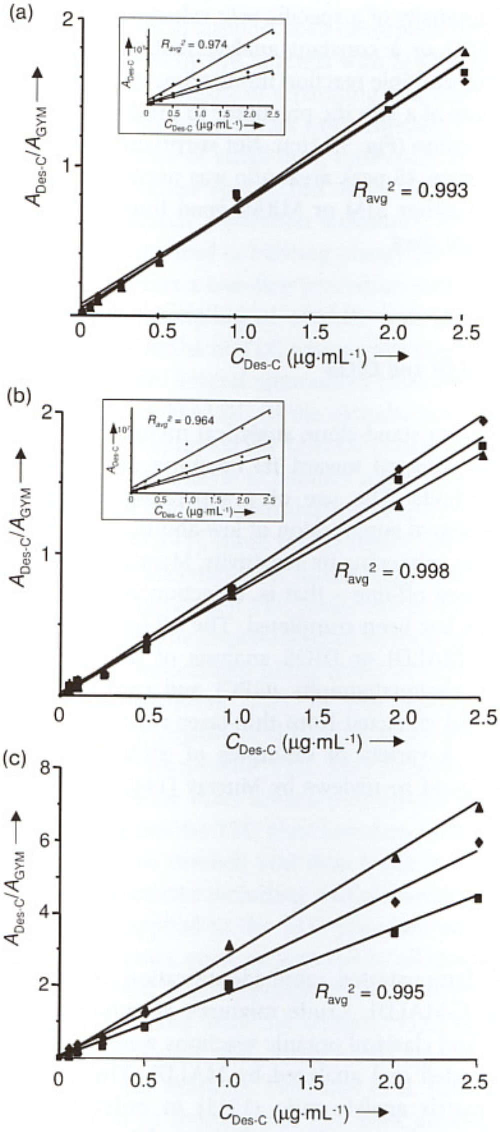


Fig. 9.11 Optimization of MALDI quantitation. Each experiment was performed in triplicate (interday variations: ▲, day 1; ◆, day 2; ■, day 3). (a) SIM detection with constant matrix concentration (inset without IS); (b) SIM detection with constant analyte : matrix ratio (inset without IS); and (c) MRM detection with constant matrix concentration. Reprinted with permission from Ref. [138]; © American Chemical Society, 2005.

conducted, encompassing screening for known and unknown spirolide analogues, quantitation of toxins in the 0.01 to 1.75 µg mL^{-1} concentration range, and preliminary structural characterization based on low-energy CID spectra. The optimization of MALDI quantitation for 13-desmethylspirolide C using gymnodimine as IS is shown in Figure 9.11. Results using selected ion monitoring (SIM, measurement of the mass spectral intensity of a specific *m/z* value) with either a set matrix concentration (Fig. 9.11(a)) or a constant analyte:matrix ratio (Fig. 9.11(b)) are contrasted with data from multiple reaction monitoring (MRM, measurement of the mass spectral intensity of a specific precursor to product ion *m/z* transition) with a set matrix concentration (Fig. 9.11(c)). Not surprisingly, greater precision was observed when the analyte:IS peak area ratio was used rather than simply the analyte peak area. Using either SIM or MRM, good linearity and r^2 values were observed on three separate days.

9.6
Separation Methods Coupled with MALDI and DIOS

Although MALDI is used primarily as a stand-alone analytical method, substantial efforts and research have been directed toward its coupling with various column and planar separation methods. The use of a separation technique prior to MS analysis greatly reduces signal suppression of low-abundance ions in the analysis of complex mixtures, thus enhancing its sensitivity. Most MALDI coupling schemes have been accomplished off-line – that is, detection is performed after the entire separation procedure has been completed. The off-line approach involves subsequent (but separate) MALDI or DIOS analysis of fractions collected from HPLC, gel-permeation chromatography (GPC) and capillary electrophoresis (CE), or spots scraped and extracted from thin-layer chromatograms and polyacrylamide gels [139–143]. A variety of examples of off-line fraction collection-MALDI analysis can be found in reviews by Murray [144] and Gusev [145].

9.6.1
TLC-MALDI

Meldal and coworkers [142] have demonstrated rapid identification of organic reaction products using off-line TLC-MALDI. Crude mixtures of peptides, glycopeptides, carbohydrate reactions, and classical organic reactions were separated on the TLC plate, scraped off, extracted and analyzed by MALDI. The authors proposed using a relatively low matrix:analyte ratio (15:1) in order to suppress matrix peaks and minimize interference. Low matrix:analyte ratios can significantly reduce matrix peaks to the point where the analyte signal become the most abundant in the spectrum. These authors also found that doping both matrix and analyte solutions with Cs^+ ions resulted in the suppression of both Na^+ and K^+ cationized peaks, which simplified the identification of LMM samples.

One novel approach in the off-line planar separation method coupled with DIOS is the use of SiNWs as a platform for the separation of molecules [38]. The capability of a SiNW to separate a sample mixture lies in its high surface-to-volume ratio and in the differences in analyte–surface interactions. When combined with an ability to support laser desorption/ionization MS, chromatographic separation followed by MS analysis with SiNWs provides a simple, inexpensive, rapid and qualitative means of separating and analyzing sample mixtures. The ability of SiNWs to separate analytes from complex mixture is demonstrated in the analysis of a mixture of small-drug molecules and endogenous analytes from human serum and mouse spinal cord tissue. With further optimization of SiNW dimensions, this planar separation technique, followed by subsequent MS analysis, is attractive compared to existing planar chromatography coupled with MALDI-MS because it offers a one-step procedure without a matrix deposition step.

The direct coupling of MALDI with planar separation yields one- and two-dimensional scans of TLC where each pixel is a mass spectrum obtained directly from the plate. Several approaches have been developed to directly couple planar separations to MALDI: (i) the introduction of a solid MALDI matrix in solution [146,147]; (ii) spraying the MALDI matrix solution onto the surface [148]; and (iii) the transfer of analyte to another membrane or support [149,150]. Although both TLC-MALDI and the optimization of the coupling protocol have been extensively reviewed [144,145], more recent reports demonstrate a continued interest in this area and advancements in methodology. Based on the availability of AP-MALDI sources, the developed TLC plates can be easily and quickly analyzed after mounting with tape. The structural identification of even incompletely separated analytes has been demonstrated using either MALDI PSD [151] or MALDI/CID with ion trap mass analyzers [152]. In one report, MALDI mass spectra allowed the detection of a mixture of chlortetracycline and tetracycline not resolved during the TLC plate development [153]. TLC coupling with AP-MALDI also offers the advantage of better mass accuracy compared to TLC-MALDI, as the changes in ion flight path from the TLC plate based on surface morphology will generate a longer detection time interval and thus lower mass resolution. In addition, a broader variety of matrixes including particle suspensions [152] or ionic liquids [153] can be directly applied to the TLC plate and analyzed on-line. Using this approach, small molecules such as antibiotics, alkaloids, anesthetics, and bacterial siderophobes (LMM Fe(III)-specific binding molecules) have been successfully separated by TLC and analyzed on-spot [152,154]. On-line TLC-MALDI quantitation has also been evaluated, with various approaches being explored for the application of IS. For example, Crecelius et al. have investigated: (i) mixing the analyte and IS prior to spotting and TLC plate development; (ii) adding IS to the mobile phase and pre-developing the TLC plate; (iii) electrospraying the IS onto the plate prior to matrix application; and (iv) mixing the IS with matrix and electrospraying the mixture. Of these four approaches, pre-developing the IS in the mobile phase was deemed to be the most successful. Results for the quantitation of piroxicam were somewhat disappointing, however, with a limited dynamic range (400–800 ng on plate) and extensive ion suppression of the analyte by IS, and IS by analyte [155].

On-line high-performance TLC (HPTLC) MALDI-FTMS at elevated pressures was recently described in the direct analysis of gangliosides from TLC plates using solid matrices [156]. Gangliosides are glycosphingolipids that contain one or more sialic acids which are easily cleaved during MS analysis. The degree of metastable fragmentation can be reduced significantly by employing a high-pressure gas pulse, with typical gas pressures ranging from 1 to 10 mbar being employed. Under this pressure regime vibrational cooling is achieved, thereby reducing ganglioside fragmentation and allowing its analysis. In addition, intact gangliosides were observed even when "hot UV MALDI matrices" were employed. HPTLC UV MALDI-MS analysis of gangliosides results in a detection limit of ~100 fmol before separation, a mass resolution of >1 : 50 000, and a mass accuracy better than 1.5 ppm. However, when IR laser and IR MALDI matrices were used, a significant fragmentation of gangliosides was observed. Moreover, the sensitivity was low due to poor incorporation of the matrices on the silica gel. A key improvement in HPTLC IR MALDI in the analysis of ganglioside mixtures from cultured Chinese hamster ovary cells was reported by Dreisewerd and coworkers [157]. In this study, a liquid matrix (glycerol) was employed for the homogeneous wetting of the silica gel, an IR laser for softer ionization and efficient desorption of gangliosides from the TLC plates, and an orthogonal TOF mass spectrometer for high mass accuracy. Results obtained from MS analysis of a mixture of gangliosides directly from HPTLC plates obtained in both positive- and negative-ion modes were characterized by high mass accuracy and low detection limit. The method also allows the simultaneous detection of various GM3 (II^3-α-Neu5Ac-LacCer) ganglioside species from analyte bands, with high relative sensitivity and lateral resolution.

A recent report from Kostiainen and co-workers utilizing ultra TLC (UTLC) separations coupled with MALDI appears to offer significant improvement over high-performance TLC approaches. The fabrication of monolithic silica UTLC plates provides 10- to 100-fold greater sensitivity than HPTLC, lower sample consumption, and faster separation. Some caveats include less resolving power due to shorter elution distances (2 cm versus 5 cm) and a smaller overall adsorption surface area available for analytes. This method has been successfully applied to the analysis, purity estimates and qualitative identification components of crude mixtures of compounds synthesized via combinatorial chemistry [158]. Although at present TLC-MALDI analysis remains only semi-quantitative in nature, its primary utility as a qualitative tool for mixture separation and structural identification is currently on the rise.

9.6.2
Capillary and Frontal Affinity Liquid Chromatography

To date, the vast majority of experiments that have coupled liquid separation techniques with MALDI have utilized off-line fraction collection. The availability of MALDI sample preparation robots has facilitated the preparation of hundreds of samples as discrete spots on the target plate in a short time frame. If the fraction collection is triggered by detection of a UV signal (or other analytical detector), then MS analysis can be focused only on the chromatographic regions which contain the most abundant analytes. In addition, fraction collection allows separation and detection of small sample volumes, with the potential to re-analyze these precious samples if necessary. Furthermore, MALDI target anchor plates can provide additional concentrating effect to further improve sensitivity.

Capillary HPLC has been hyphenated with MALDI-MS for nanoscale screening of single-bead combinatorial libraries [159]. A small test library of nine compounds showed optimum results using DHB as matrix with on-line mixing of HPLC effluent and matrix solution followed by ESD. Seven corticosteroids have been identified using 2,4-dinitrophenylhydrazine as both derivatizing agent and matrix. Derivatization of the analytes was accomplished on-line during ESD and drying time [160].

Off-line collection of LC effluent can also be accomplished into continuous channels or "tracks" on the MALDI target plate. In this manner, the ion signals from rastering the laser along this channel can be used to generate a reconstructed chromatogram from a more continuous process than spotting discrete fractions. This approach has been successfully applied to the screening of compound libraries using frontal affinity chromatography (FAC) [161]. FAC relies upon continuous infusion of a known concentration of compound (rather than a single injection as in HPLC) to drive equilibration of the ligand between free and bound states with an immobilized protein stationary phase. The breakthrough time of the compound should correspond to the ligand affinity for the immobilized protein. Potent inhibitors should exhibit greater affinity and thus be retained longer on the protein stationary phase. Coupling MALDI with FAC circumvents some inherent problems with FAC ESI-MS as the need for high ionic strength eluents in FAC causes ion suppression in ESI. FAC MALDI-MS was applied to the screening of small molecules against entrapped dihydrofolate reductase (DHFR). Figure 9.12 shows FAC MALDI-MS/MS traces from columns with and without DHFR. Only results from the first two tracks on the target plate are shown; once all the analyte signals reach a plateau further analysis is unnecessary. Potent inhibitors trimethoprim and pyrimethamine exhibited significantly greater affinity (and thus retention) than fluorescein, glucosamine and folic acid. Results using MALDI were comparable to those obtained by ESI, and offer greater throughput and good sensitivity.

Although an off-line approach simplifies the coupling and allows for the separation and MALDI-MS steps to occur independently, this provides only the mass spectral data for several discrete times (HPLC, GPC, and CE) or spots (TLC and gels). A few experimental approaches for continuous monitoring of the entire separation include: (i) aerosol MALDI liquid introduction [162–164], analogous to the particle beam interface; (ii) a continuous-flow MALDI probe with liquid matrix or continuous solid matrix crystallization [165–170], analogous to continuous-flow FAB; and (iii) rotational ball or wheel MALDI interfaces [171,172], analogous to a moving-belt interface. Although the on-line coupling of MALDI and liquid column separation is currently in the experimental stages, it may yet revolutionize the MALDI-MS field and have a major impact on the characterization of LMM samples in complex mixtures or biological and environmental matrices.

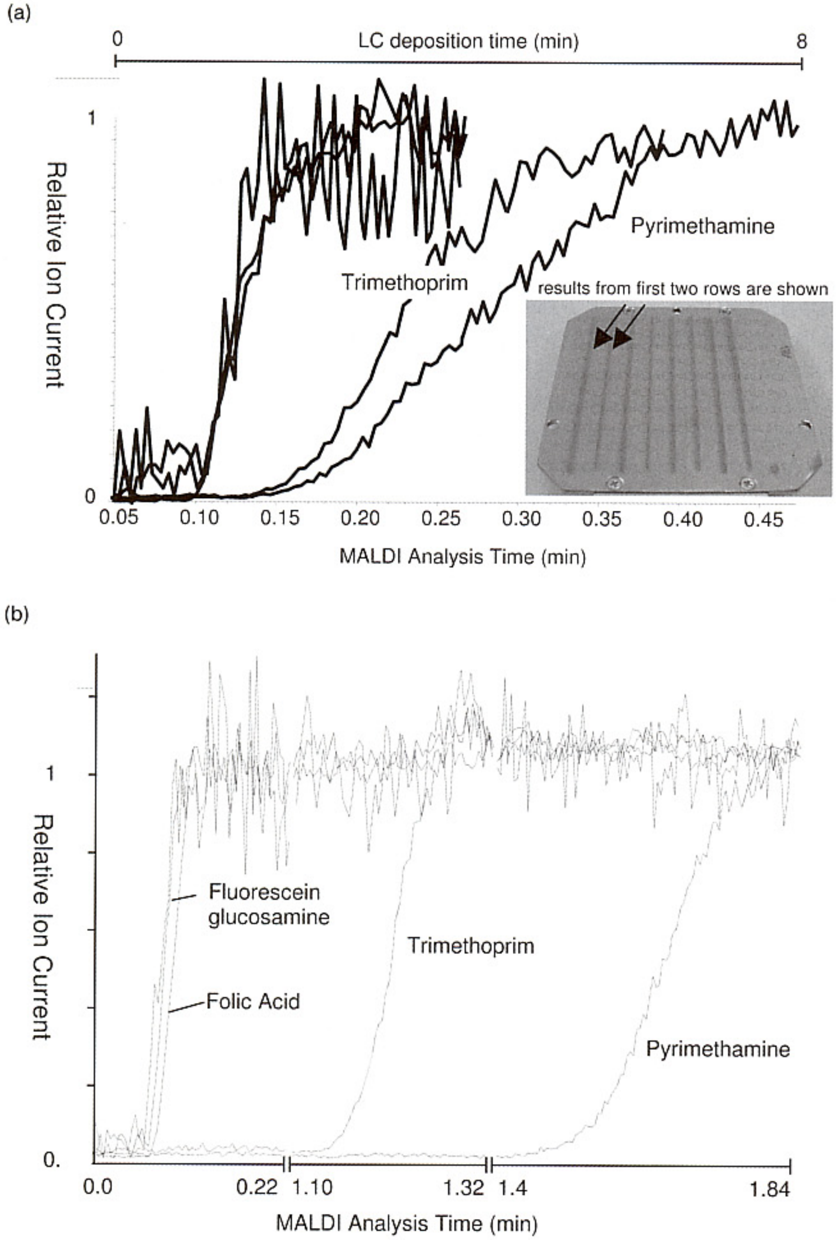


Fig. 9.12 FAC MALDI-MS/MS traces obtained using protein-loaded and blank polymer-coated macroporous silica monolithic columns. (A) Blank column containing no protein. (B) Column containing 25 pmol DHFR (initial loading) showing breakthrough of *N*-acetylglucosamine, fluorescein, and folic acid at early times, then trimethoprim, and finally pyrimethamine. All compounds were infused at 50 nM. All traces are normalized to the maximum signal obtained after compound breakthrough. Note that the MALDI analysis time is 19-fold faster than the LC deposition time. All FAC traces were obtained using a fast laser translation speed, 3.8 mm s^{-1}) and represent the average of five re-runs over a given sample region. Reprinted with permission from Ref. [161]; © American Chemical Society, 2005.

9.7
Conclusions

The wide variety of both numerous and diverse applications described in this chapter clearly demonstrates the utility and future potential of MALDI for both qualitative and quantitative characterization of LMM compounds. Many new instrumentation and sample preparation techniques are now available for automated and painless MALDI characterization. Arguably, sample preparation from complex milieu such as blood still requires considerable attention and effort. The sample clean-up procedures prior to MALDI analysis need not be more complicated or lengthy than those conducted for ESI-MS analysis. Realistically, problems such as shot-to-shot and sample-to-sample reproducibility still exist, but can be overcome by the techniques discussed earlier. Other criticisms of quantitative MALDI such as poor sensitivity, limited linear range and imprecision (higher RSD) are primarily compound-dependent and have been significantly improved by instrumental advancements.

The greatest potential for utilizing the full analytical power of MALDI in the low mass range exists for mixtures of both high- and low-molecular weight components, as well as both polar and nonpolar compounds. At present, mixtures require tedious optimization of the type of matrix and either sequential analysis by MALDI or complementary analysis by electrospray MS. Novel sample preparation approaches such as SALDI or DIOS, which simplify the process, serve as an extraction step during sample preparation, and remove the matrix altogether, are being increasingly applied to more complex problems. We can envision exciting opportunities for the application of MALDI to simultaneous analysis of biomarkers in biological media, including both large molecules (proteins) and small molecules such as pharmaceutical compounds or glucose.

The somewhat surprising number and scope of publications on MALDI characterization of LMM compounds not only reinforces the utility and practical applicability of this technique, but also indicates the substantial efforts being made by several research groups. These efforts are transforming what might be pigeonholed as a "high-molecular mass biomolecule only" analytical technique into a flexible analytical tool.

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Abbreviations/Acronyms

α-CHCA	α-cyano-4-hydroxycinnamic acid
AChE	acetylcholine esterase

AP/MALDI	atmospheric pressure/matrix-assisted laser desorption/ionization
BOC	*t*-butyloxycarbonyl
CE	capillary electrophoresis
CID	collision-induced dissociation
CNT	carbon nanotube
CsA	cyclosporine A
CTAB	cetrimonium bromide
DHB	2,5-dihydroxy benzoic acid
DHBB	2,5-dihydroxy benzoic acid butylamine
DHB-Py	2,5-dihydroxy benzoic acid pyridine
DIOS	desorption/ionization on silicon
DOPA	3,4-dihydroxyphenylalanine
DOPAQ	dopaquinone
ESD	electrospray sample deposition
ESI	electrospray ionization
FAC	frontal affinity chromatography
GM3	II^3-α-Neu5Ac-LacCer
HABA	2-(4-hydroxyphenylazo)benzoic acid
HPLC	high-performance liquid chromatography
IR	infrared
IS	internal standard
LacNac	*N*-acetyllactosamine
LDI	laser desorption ionization
LMM	low molecular mass
MALDI	matrix-assisted laser desorption/ionization
MET	2-methoxy-*N*-[(1*R*)-1-phenylethyl]acetamide
MRM	multiple reaction monitoring
MS	mass spectrometry
MSE	matrix suppression effect
NBA	3-nitrobenzyl alcohol
NMR	nuclear magnetic resonance
NPE	nonphenylethoxylates
NPOE	nitrophenyl octyl ether
OBOC	one-bead–one-compound
PEA	rac-a-phenylethylamine
PEG	polyethylene glycol
PMMA	polymethylmethacrylate
PSD	post source decay
pSi	porous silicon
PTSA	*p*-toluenensulfonic acid
RSD	relative standard deviation
SALDI	surface-assisted laser desorption/ionization
SIM	single ion monitoring
SiNW	silicon nanowire
SLE	solid-liquid extraction

S/N	signal-to-noise
SPE	solid-phase extraction
SPIE	solid-phase immunoextraction
TAC	tacrolimus
TCNQ	7,7,8,8-tetraccyanoquinodemethane
THF	tetrahydrofuran
TLC	thin-layer chromatography
TOF	time-of-flight
UTLC	ultra thin-layer chromatography
UV	ultraviolet
VLS	vapor liquid solid

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