BIOMEDICAL APPLICATION OF MALDI MASS SPECTROMETRY FOR SMALL-MOLECULE ANALYSIS

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Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is an emerging analytical tool for the analysis of molecules with molar masses below 1,000 Da; that is, small molecules. This technique offers rapid analysis, high sensitivity, low sample consumption, a relative high tolerance towards salts and buffers, and the possibility to store sample on the target plate. The successful application of the technique is, however, hampered by low molecular weight (LMW) matrix-derived interference signals and by poor reproducibility of signal intensities during quantitative analyses. In this review, we focus on the biomedical application of MALDI-MS for the analysis of small molecules and discuss its favorable properties and its challenges as well as strategies to improve the performance of the technique. Furthermore, practical aspects and applications are presented. © 2010 Wiley Periodicals, Inc., Mass Spec Rev 30:101–120, 2011

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I. INTRODUCTION

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has gained a prominent role in the analysis of biopolymers such as proteins, peptides, oligonucleotides, and oligosaccharides. Yet, this technique is not commonly applied to the analysis of molecules with molar masses below 1,000 Da, which are called small molecules or LMW molecules. For this application, MALDI-MS has found strong competition from the combination of liquid chromatography (LC) with atmospheric pressure ionization MS (LC–MS), in particular from the combination of LC and electrospray ionization (ESI) MS. Unambiguous annotation of MALDI mass spectra of LMW compounds can be complicated due to the presence of matrix-derived peaks in the low mass range; that is, below *m/z* 1,000. The poor reproducibilities of signal intensities that have frequently been reported for MALDI-MS hampered its application to quantitative analysis.

Notwithstanding the above, MALDI-MS has some intrinsic properties that favor its use for the analysis of LMW molecules such as its high tolerance towards salts and buffers, rapid analyses, its high absolute sensitivity, the small amount of sample consumed during analysis, and the possibility to store samples on a target plate for longer periods of time. These intrinsic properties of MALDI-MS have inspired scientists to find ways to improve the performance of this technique for the analysis of LMW compounds. A variety of approaches have been described to decrease or circumvent matrix-related peaks, such as the use of high molecular weight (MW) matrices, alternative sample-preparation procedures, and application of tandem mass spectrometry (MS/MS). Reproducibility of signal intensities can be improved, for example, with internal standards, and by procedures to enhance homogeneous crystallization of matrix and sample. Recently, a MALDI-triple quadrupole mass spectrometer has been introduced, which is the first MALDI mass spectrometer developed specifically for the quantitative analysis of LMW compounds. Although MALDI-MS is gaining acceptance as an analytical tool for the analysis of LMW compounds, application of the technique to this field is still limited.

In this review, we discuss the biomedical application of MALDI-MS to the analysis of LMW compounds. The review published in 2002 (Cohen & Gusev, 2002) also deals with the analysis of small molecules by MALDI-MS and serves as an excellent background to the present report. We first discuss the properties of MALDI-MS that favor its application to the analysis of LMW molecules, which are the high-throughput analysis, its relative insensitivity for ion suppression in complex and clinical samples, the high absolute sensitivity, the low sample consumption, and the storage of samples on target plates. The two main challenges of MALDI-MS for small-molecule analysis are discussed, which are the interfering signals derived from the MALDI matrix and the poor reproducibility of signal intensities. Strategies are presented to overcome these challenges. Practical aspects of small-molecule analysis are discussed, which are cationization agents, MS and MS/MS strategies, and preparation of biological samples. We focus on the application of MALDI-MS to the quantitative analysis of drugs, metabolomics, and

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monitoring of enzymatic conversion of small molecules. Finally, we present our thoughts on the future directions of MALDI-MS to the analysis of LMW compounds.

II. CHOICE OF MALDI FOR SMALL-MOLECULE ANALYSIS

A. High-Throughput Analysis

For MALDI-MS, offline sample-preparation techniques such as solid-phase extraction (SPE), liquid–liquid extraction (LLE), and protein precipitation (PP) provide sufficiently clean extracts of biological samples for direct analysis. These sample-preparation techniques are performed offline (i.e., sample preparation and MALDI measurements are completely de-coupled) and are highly suited for the simultaneous preparation of relatively large sets of samples. To increase the throughput of sample preparation prior to MALDI-MS, these relatively easy sample extraction methods as well as spotting of samples onto target plates can readily be automated with 96- or 384-well formats. MALDI-MS is less susceptible to ion suppression compared to ESI-MS (Yang et al., 2007), and a LC step is normally not needed to prepare samples of biological origin.

The major contributor to the total analysis time in LC–MS is the LC step. For quantitative analysis of pharmaceutical compounds in biological samples, the LC step still takes several minutes, even with new LC developments such as monolithic columns and ultra-pressure LC. In MALDI-MS, however, the sample analysis time is primarily determined by the number of laser shots needed to generate an average mass spectrum of high quality and the repetition rate of the laser used. Dwell time of the ions does not contribute significantly to the analysis time, although the detection time of ions can take second(s) per scan in Fourier-transform ion cyclotron resonance (FTICR) MS and Orbitrap MS. With a high repetition rate laser that fires at 1,000–2,000 Hz, an averaged mass spectrum of high quality can be obtained in a few seconds with MALDI-time-of-flight (TOF) MS or MALDI-triple quadrupole MS (Hatsis et al., 2003; McLean, Russell, & Russell, 2003; Moskovets et al., 2006; Rathore et al., 2008). For quantitative analysis of, for instance, HIV protease inhibitors, analysis times for a single spot on the target plate have been reported of ∼3 min for MALDI-FTICR MS (20 Hz laser), ∼30 sec for MALDI-TOF MS (50 Hz laser), and ∼5 sec for MALDI-triple quadrupole MS (1,000 Hz laser; van Kampen et al., 2006, 2008a,b, 2009b). Recently, an analysis time of 1.75 min for an entire 384-well target plate was reported for an assay to screen for small-molecule inhibitors of enzymes with MALDI-triple quadrupole MS (Rathore et al., 2008). When the time required for vacuum pump down was included, the total analysis time for a single target plate was still just under 3.5 min.

B. Ion Suppression

In MALDI-MS, strong signals can be obtained for analytes that are present in complex biological samples and in samples that contain salts and buffers that are frequently used in biomedical research. Various studies report in detail on the effect of various buffers and salts on the MALDI MS analysis of biopolymers such as proteins, peptides, and DNA (Mock, Sutton, & Cottrell, 1992; Kallweit et al., 1996; Shaler et al., 1996; Yao et al., 1998; Amini et al., 2000; Bajuk, Gluch, & Michalak, 2001). Such studies have not yet been performed for pharmaceutical compounds. Some general statements on contaminants and tolerance limits can be made: (1) contaminants can affect analyte signals by inducing extensive, concentration-dependent adduct formation such as salts. Also, crystallization of the sample might be impaired when contaminants such as Tris and urea are present (Shaler et al., 1996). (2) The tolerance limits for a contaminant can depend on the type of analyte under investigation. For example, the tolerance limit for alkali-metal salt impurities in protein and peptide analysis is ∼1 M, whereas it is 10^{-2} M for DNA (Shaler et al., 1996). On the other hand, the tolerance limit for the buffer Tris is comparable for proteins, peptides, and DNA (∼0.5 M) (Kallweit et al., 1996; Shaler et al., 1996; Yao et al., 1998). (3) The tolerance limit depends on the type of contaminant. In particular, phosphate buffers are known to quench analyte signals (Mock, Sutton, & Cottrell, 1992; Kallweit et al., 1996). (4) The effect of buffers on the sample crystallization is matrix-dependent. For example, Kallweit et al. (1996) showed that crystallization of 2,5-dihydroxybenzoic acid (2,5-DHB) and in particular of 2,6-dihydroxyacetophenone is more affected by commonly used buffers compared to sinapinic acid (SA). (5) Sample preparation might influence tolerance limits. For example, buffer tolerances are increased significantly when higher matrix-to-analyte ratios are used (Yao et al., 1998), and additives such as diammoniumhydrogencitrate (DAHC) can improve the tolerance of the matrices against the buffers and salt adduct formation (Kallweit et al., 1996). Recently, a comprehensive database has been compiled of known interferences and background-ions in modern MS (Keller et al., 2008).

C. Sample Consumption and Sensitivity

Sample volumes used for bioanalysis are lower for MALDI-MS than for LC–MS. Typically, 0.5–1 μL of sample/matrix mixture is deposited on a target plate for MALDI-MS analysis. For LC–MS, sample volumes of typically 25–50 μL are injected into the LC, which separates and concentrates the compounds before ionization. Thus, in LC–MS, larger sample volumes can be used and the whole sample is consumed during analysis. In MALDI-MS, only a small portion of the spotted sample is consumed during analysis. Approximately 4% of a sample is ablated during MALDI-triple quadrupole MS analysis with a 1,000 Hz laser that fires 3,000–4,000 shots per sample (Sleno and Volmer, 2005a). However, the smaller sample volumes that can be used in MALDI-MS, and the low sample consumption during measurements, are compensated for by the high sensitivity of MALDI-MS. Sleno and Volmer (2005a) calculated that, at the limit of detection of ramipril (0.5 nM), an average of 6.5 zmol of this compound was ablated per laser shot. Direct comparison of analyte signal in relation to sample consumption between MALDI-triple quadrupole MS and LC–ESI-triple quadrupole MS showed that MALDI-MS is approximately 100 times more efficient than ESI-MS (Gobey et al., 2005). Thus, in MALDI-MS, smaller volumes of samples can be used for analysis, smaller

amounts of the samples are consumed during analysis, but higher efficiencies are obtained. Application of MALDI-triple quadrupole MS has shown that the intrinsic differences between MALDI-MS and LC–MS described above approximately cancel, and in practice therefore, the limits of quantification obtained by MALDI-MS and ESI-MS are approximately comparable (Volmer et al., 2007).

D. Storage of Samples on Target Plates

The low sample consumption during MALDI-MS measurements allows storage and reanalysis of the samples. Reanalysis of a tryptic digest after 1-month storage on a target plate in a closed container showed that peak intensities were not affected (Dekker et al., 2007). For HIV protease inhibitors, we have shown that neither precision, accuracy, nor the limit of quantification (LOQ) were affected when calibrators and quality control samples for HIV protease inhibitors in plasma were stored in a closed container for a month under ambient conditions (van Kampen et al., 2009b). Target plates that contain the co-crystallized sample/matrix mixtures should be stored in the dark, because MALDI matrices are photoactive. Furthermore, storage under an inert gas can prevent sample degradation over time; for example, oxidation of the analyte might occur under ambient conditions. Sample storage on a target plate also allows researchers to obtain mass spectra of the same sample with different types of MALDI mass spectrometers. Dekker et al. (2007) showed that protein identification of a tryptic digest improved when accurate mass measurements of the precursor ions obtained by MALDI-FTICR were combined with the MS/MS data obtained by MALDI-TOF.

III. CHALLENGES OF MALDI-MS FOR SMALL-MOLECULE ANALYSIS: CHEMICAL NOISE

A. Origin of Chemical Noise in MALDI

Matrix-assisted laser desorption/ionization (MALDI) MS spectra are characterized by various strong signals that derive from the matrix such as signals from the protonated matrix ($[\text{matrix} + \text{H}]^+$ for positive-ion MALDI), fragment ions (e.g., $[\text{matrix} - \text{H}_2\text{O} + \text{H}]^+$), and clusters thereof (e.g., $[\text{matrix}_n + \text{H}]^+$, $[\text{matrix}_n + \text{alkali}]^+$, and $[\text{matrix}_n - \text{H}_2\text{O}_n + \text{H}]^+$). For example, 2,5-DHB ($C_7H_6O_4$) produces, after laser irradiation, the series of $(C_7H_5O_4Na)_nNa^+$ ions in admixture with other adducts, even in the absence of extraneous sodium ions (Gouw et al., 2002). Typically, these intense matrix signals are observed up to m/z 500. At higher m/z values, the number of these matrix signals, as well as their intensity, is generally less. In addition to the various intense matrix signals, a ''bumpy'' baseline is observed over the entire m/z range. Krutchinsky and Chait (2002) further investigated the nature of this baseline by performing MALDI-ion trap MS/MS experiments and found that essentially every m/z value of the baseline is derived from matrix ions (see Fig. 1). Furthermore, they found that these matrix ions have lower activation energies for fragmentation than the analyte ions. Thus, by performing broadband collisional activation below the threshold for analyte fragmentation, the chemical noise levels were decreased to thereby improve the signal-to-noise ratio (S/N) of the analyte signal. An important consequence of the lower activation energies for matrix-derived signals is that they will have decomposed after very long ion life times. This important property, which is a direct consequence of RRKM theory, can be exploited in vacuum MALDI-FTICR, where matrix-derived signals are correspondingly absent, or greatly reduced (see Section IIIC).

B. High Molecular Weight Matrices

One way to avoid the interfering matrix signals in the low m/z range is to choose a matrix whose molar mass exceeds that of the analyte. In this way, the matrix-derived peaks in the low mass range result only from dissociation of the matrix or from matrix impurities but not from the molecular ion of the matrix, matrix adducts, and matrix clusters. Srinivasan et al. (1999) reported that some porphyrins could act as auto-matrices, and various porphyrins have been tested as matrix in MALDI-MS (Jones, Lamb, & Lim, 1995; Chen & Ling, 2002). In particular, meso-tetrakis(pentafluorophenyl)porphyrin (F20TPP; MW 974) appears to be a useful matrix for the analysis of small molecules with MALDI-TOF MS (Ayorinde et al., 1999; Ayorinde, Garvin, & Saeed, 2000; Hlongwane et al., 2001; Ayorinde, Bezabeh, & Delves, 2003; van Kampen et al., 2006, 2007; Yu et al., 2006; Kosanam et al., 2007). Ayorinde et al. (1999) compared the use of α-cyano-hydroxycinnamic acid (CHCA) and F20TPP for the analysis of alkylphenol ethoxylates and found that F20TPP generated spectra with significantly less matrix interference in the low mass range (see Fig. 2). Further studies showed that F20TPP is a suitable matrix for the determination and quantification of fatty acids (Ayorinde, Garvin, & Saeed, 2000; Hlongwane et al., 2001; Yu et al., 2006), and to determine the constituents of non-alcoholic beverages such as sugars, ascorbic acid, and citric acid (Ayorinde, Bezabeh, & Delves, 2003). F20TPP is also a useful matrix for MALDI-TOF analysis of several pharmaceutical compounds such as HIV protease inhibitors, reverse transcriptase inhibitors, and antibiotics (van Kampen et al., 2006). In addition, the matrix can be used for the quantitative analysis of lopinavir and ritonavir in cell lysates (van Kampen et al., 2006, 2007). Limits of quantification were 25 fmol for lopinavir and 16 fmol for ritonavir, and precisions and accuracies were within the ±20/15 FDA criteria (i.e., precision and accuracy below 20% CV and 20% deviation, respectively, at the LOQ, and below 15% CV and 15% deviation, respectively, at higher concentrations). F20TPP has also been used for the quantitative analysis of doping agents in urine with a MALDI-ion trap mass spectrometer (Kosanam et al., 2007). Testosterone, nandrolone, betametasone, boldenone, and trenbolone were quantified down to 0.1 ng/mL. Precisions and accuracies fulfilled the ±20/15 criteria. The light-absorbing, electrically conductive polymer polythiophene has been used as a high MW matrix for negative-ion MALDI-MS (Soltzberg & Patel, 2004). Buckminsterfullerene (C_{60}; MW 720) has also been used as a high MW matrix for the analysis of small molecules. This approach was used to screen for diuretics in urine (Huang et al., 1999), and detection limits of 0.1–1 μg/mL were obtained. C_{60} is insoluble in many solvents, including water, and this property might

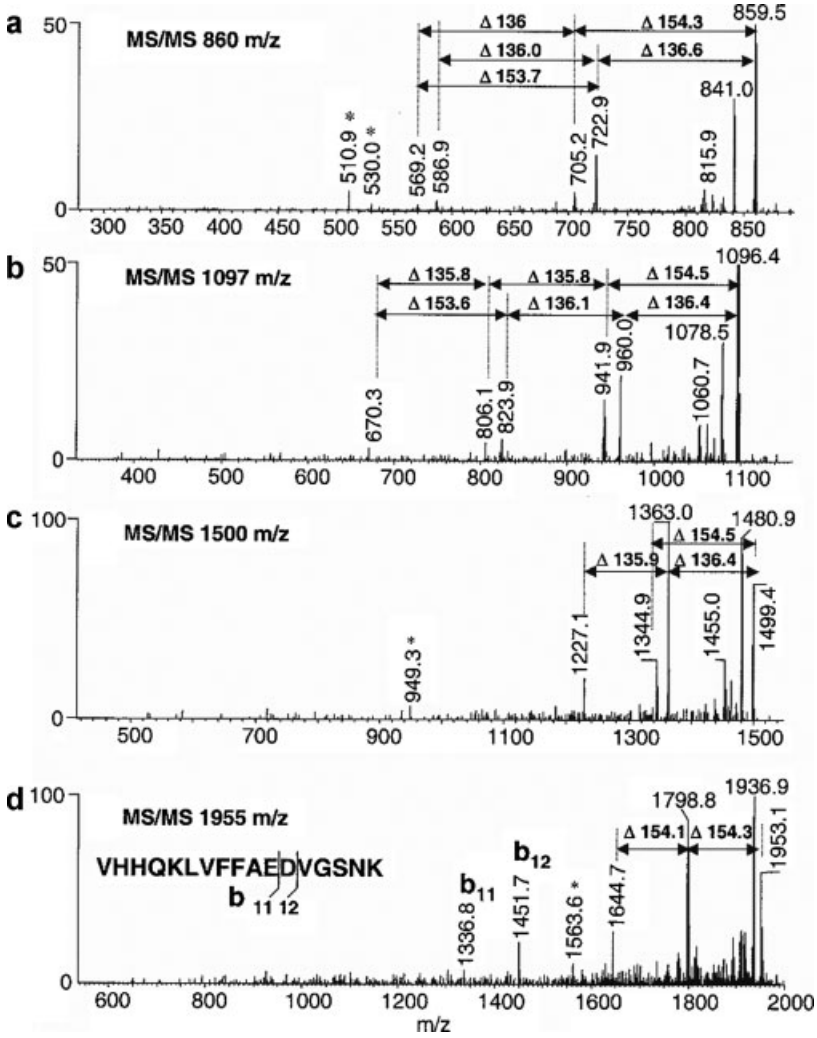

FIGURE 1. Origin of chemical noise in MALDI. MALDI-MS/MS mass spectra obtained from a selection of *m*/*z* values where no analyte signals are expected (**a**–**c**), as well as one *m*/*z* value (1,955) where a peptide signal is expected (**d**). Characteristic losses of intact molecules of 2,5-DHB (154 Da) and molecules of 2,5-DHB with eliminated water (136 Da) from the precursor are indicated. The asterisk indicates unexplained ion peaks. Reprinted with permission from Krutchinsky and Chait (2002), copyright 2002 Elsevier Science B.V. http://www.sciencedirect.com/science/journal/10440305.

complicate sample preparation. Derivatives of fullerene such as hexa(sulfonbutyl)fullerene are water soluble and can be used to selectively precipitate analytes in aqueous solutions, which are then analyzed with MALDI-MS without the addition of a matrix (Shiea et al., 2003).

C. MALDI-FTICR

When MALDI-FTICR is used for small-molecule analysis, a LMW matrix might be preferred over a high MW matrix (van Kampen et al., 2008a). Attempts to use the high MW matrix F20TPP for MALDI-FTICR analysis showed extensive fragmentation of F20TPP, which resulted in matrix-derived peaks in the low mass range. In contrast, the use of 2,5-DHB for MALDI-FTICR analysis gives spectra that are relatively free of matrix adducts. The opposite is observed when these matrices are used for MALDI-TOF analysis. These seemingly contrasting results can be explained by the quasi-equilibrium theory, which is a model to describe the unimolecular decomposition of ions. In this model, the rate constant for dissociation of ions is a function of excess energy (Gross, 2004). The rate constants have the dimension of sec^{-1}; that is, the process can happen that often per second. For example, if we postulate a rate constant of $10^3\,sec^{-1}$ for F20TPP, then F20TPP ions dissociate on average in 10^{-3} sec. The time between ionization and detection of ions varies among mass analyzers. Typically, it takes 10^{-4} sec to detect ions in a TOF or a quadrupole, and 1 sec in FTICR. Thus, with the postulated rate constant, the F20TPP ions formed in the MALDI process easily survive the TOF and quadrupole time scale but not the FTICR time scale. In the same vein, non-covalent matrix adducts of a LMW matrix such as 2,5-DHB have

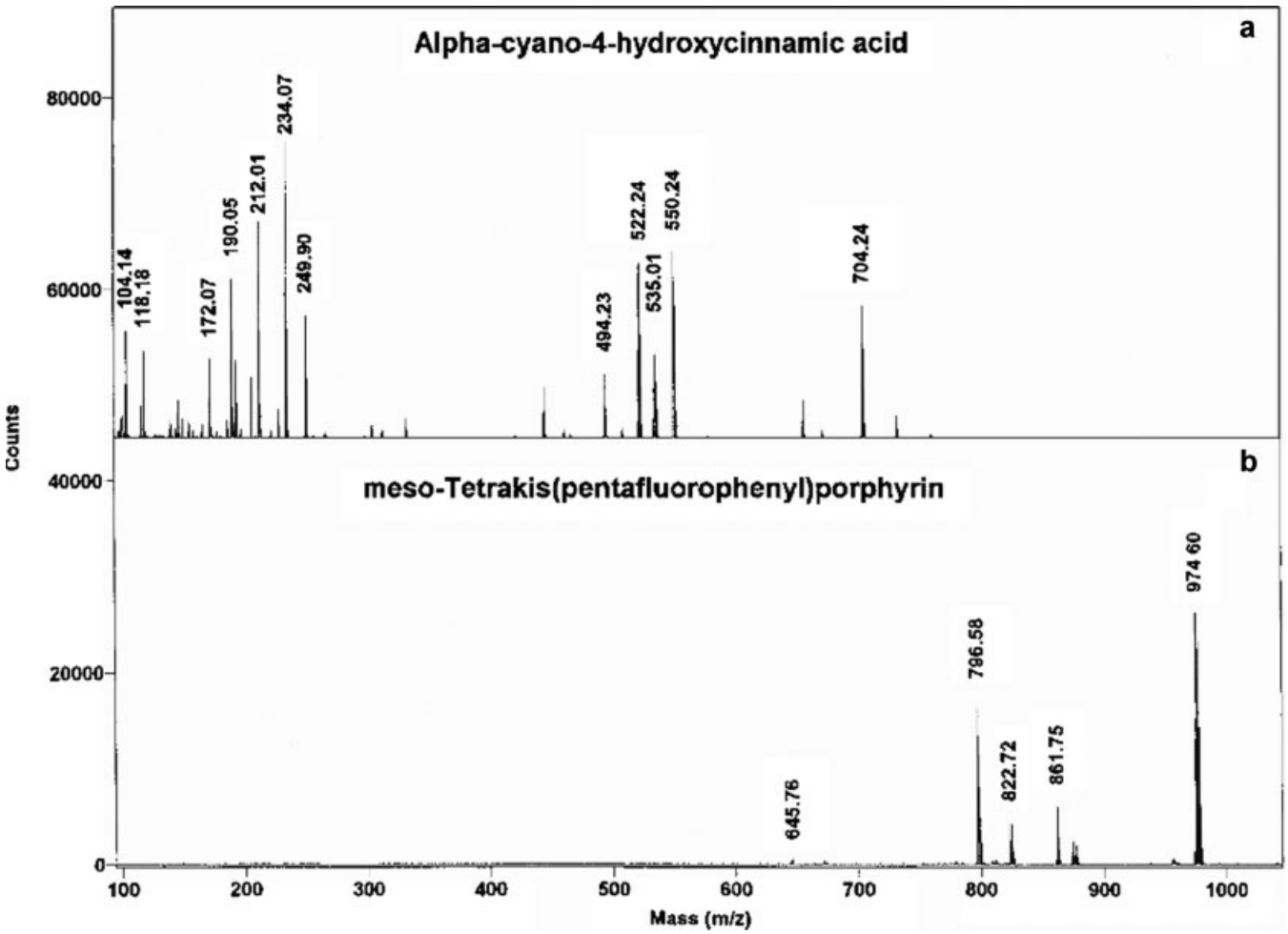

FIGURE 2. High molecular weight porphyrin matrix. MALDI-TOF mass spectra of the matrix CHCA (**a**) and the matrix F20TPP (**b**) without the addition of analytes. Reprinted with permission from Ayorinde et al. (1999), copyright 2009 John Wiley & Sons, Ltd.

lower activation energies for dissociation compared to analyte ions (Krutchinsky & Chait, 2002); therefore, these matrix-derived ions dissociate on the FTICR time scale to produce a mass spectrum free of interfering matrix peaks even in the low mass range.

D. Matrix-Free Approaches

Matrix-free LDI is an approach in which a sample is placed on a photoactive but non-desorbable support and analyzed without any matrix. For the purpose of this review, we consider this approach as a form of MALDI-MS, although no matrix is used and some differences exist in the mechanism of ion formation. The main advantage of this approach is that little or no signals from the modified surfaces are observed in the mass spectrum. In addition, sample preparation is simplified because the analyte does not need to be mixed and/or co-crystallized with a matrix. The first matrix-free MALDI method was introduced by Wei, Buriak, and Siuzdak (1999), who used porous silicon surfaces (desorption ionization on porous silicon—DIOS). The porous silicon surfaces are easily oxidized to allow for chemical modification of the surfaces. Silylation of oxidized porous silicon has been shown to improve sensitivity, shelf-life, ease of modification, and analyte specificity (Trauger et al., 2004). Furthermore, these modified surfaces can be used to selectively capture analytes from a complex mixture (Cohen, Go, & Siuzdak, 2007). Silicon nanowires have also been used for matrix-free LDI (Kang et al., 2005). Chemical modification of silicon nanowires can also improve performance. Other types of nanostructures such as carbon nanotubes and mesoporous tungsten titanium oxide surfaces have been used for matrix-free LDI. Reviews of matrix-free MALDI approaches can be found in the literature (Cohen, Go, & Siuzdak, 2007; Najam-ul-Haq et al., 2007; Petterson, 2007).

E. Additives

Additives such as the cationic surfactant cetyltrimethylammonium bromide (CTAB) can significantly decrease, or even

eliminate, matrix-derived signals of CHCA (Guo et al., 2002). High-quality spectra were obtained with a CHCA-to-CTAB molar ratio of 1,000:1. However, detection limits of the analytes were approximately one order of magnitude worse. Su, Liu, and Lin (2005) used the CHCA/CTAB matrix to screen for the drugs amphetamine, metamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, caffeine, ketamine, and tramadol in clandestine tablets. Limits of detection ranged from 2 to 10 ng/mL. Results were comparable to GC–MS. GC–MS, however, required derivatization of the analytes and additional sample handling. For MALDI-TOF MS, tablet powder was simply dissolved in methanol and directly used for analysis. Grant and Helleur (2008a,b) showed that CTAB can also be used to suppress matrix-derived signals of 2,4,6-trihydroxyacetophenone (THAP), 2,5-DHB, SA, and dithranol. The THAP/CTAB matrix was used to analyze flavonoids in berry extracts (Grant and Helleur, 2008b), and the CHCA/CTAB matrix was used to analyze vitamins and caffeine in energy drinks (Grant and Helleur, 2008a). In addition to suppression of matrix-derived signals, CTAB also improved the resolution and the quantitative performance; that is, the % CV decreased and the linearity improved. Other surfactants can also be used to decrease the number of matrix-derived signals of CHCA (Grant & Helleur, 2007); various cationic surfactants were tested, and CTAB showed in general the best performance in terms of strong analyte signals and suppressed matrix signals. The anionic surfactant sodium dodecyl sulfate was useful to analyze small peptides, and the neutral surfactant Brij 30 was useful to analyze caffeine. Cyclodextrin has been used to suppress alkali-metal ion adducts, cluster formation, and fragmentation of matrix and analyte (Yamaguchi et al., 2008). Washing CHCA crystallized samples with ammonium-containing buffers eliminated the alkali-metal matrix cluster signals and improved intensities of peptides. Ammonium salts can also be used as an additive in the matrix solution, although a weaker effect is observed (Smirnov et al., 2004).

Suppression of matrix-related peaks can also be achieved by mixing two conventional MALDI matrices (Guo & He, 2007). A mixture of the acidic matrix CHCA and the basic matrix 9-aminoacridine (9-AA) resulted predominantly in the formation of $[\text{CHCA} - \text{H}]^-$ and $[\text{9-AA} + \text{H}]^+$, and an overall reduction in matrix clusters. The binary matrix improved the S/N of the analytes; however, this improvement was only observed when the pK_a values of the analytes were substantially different than those of the matrices; analyte protonation was not affected by 9-AA when compounds were tested with more basic pK_a values than 9-AA. In that case, 9-AA reduced the amount of CHCA-related peaks to thereby improve the S/N of the analytes. For the negative-ion mode, the reverse was observed; analyte deprotonation was not affected when compounds were used that were better proton donors than CHCA.

F. Matrix-Suppression Effect

The matrix-suppression effect (MSE) is attributed to a depletion of primary matrix ions by neutral analytes via a secondary ion-molecule reaction in the plume; that is, suppression of matrix peaks is observed when sufficient analyte is present to react with all matrix ions. The MSE is observed for the disappearance of all types of matrix ions; for example, $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$ (Knochenmuss et al., 1996, 1998, 2000). McCombie and Knochenmuss (2004) tested the utility of MSE for routine MALDI analysis of small molecules. The most important and easy adjustable factors in MSE were the molar ratio of analyte and matrix in the sample, and the laser intensity (Knochenmuss et al., 1996, 1998). The highest MSE was achieved at low laser intensities and high analyte concentrations. Analyte/matrix ratios of 1:10 or less were needed to induce MSE for small-molecule analysis. The extent of MSE was related to the type of analyte under investigation. For proton transfer to the analyte, the MSE was greater for analytes with higher gas-phase basicity than for analytes with lower gas-phase basicity. The utility of MSE has been investigated for metabolome analysis by MALDI-MS (Vaidyanathan, Gaskell, & Goodacre, 2006). MSE was obtained for a cocktail of 30 metabolites that included amino acids, organic acids, and other metabolites. Furthermore, it was possible to detect the metabolites spiked in a microbial extract. However, suppression of analytes was also observed when the concentration of the analyte was increased in relation to the other metabolites.

G. Tandem Mass Spectrometry

An elegant way to circumvent the problem of the intense matrix-related peaks as well as the noisy baseline is to perform MS/MS. In this way, commonly employed matrices such as CHCA and 2,5-DHB can be used without further sample-preparation adaptations to decrease matrix-related noise. MS/MS is widely used for bioanalysis with LC–MS/MS, and triple quadrupoles are regarded as the cornerstone mass analyzer for targeted quantitative analysis of small molecules. Recently, a MALDI-triple quadrupole mass spectrometer was developed (Hatsis et al., 2003; Corr et al., 2006). In the selected reaction-monitoring (SRM) mode of a triple quadrupole instrument, isobaric matrix ion interferences can be removed by monitoring only compound-specific precursor/product ion transitions. Other mass analyzers such as ion traps and quadrupole TOFs (QqTOFs) have been used for MS/MS of small molecules. A triple quadrupole instrument, however, reaches a higher duty cycle of almost 100%, which results in highly sensitive measurements. Direct comparison of MALDI-QqTOF and MALDI-triple quadrupole showed indeed that MALDI-triple quadrupole offers ~10-fold better detection limits than MALDI-QqTOF (Hatsis et al., 2003). Furthermore, the dynamic ranges of the calibration curves obtained with MALDI-triple quadrupole were ~1 order of magnitude larger than those obtained with MALDI-QqTOF. This improvement in dynamic range was attributed to the higher sensitivity of the triple quadrupole that thereby extended the linearity of the calibration curves at the low end.

IV. REPRODUCIBILITY IN MALDI

A. Origin of Poor Precision in MALDI

Quantitative analysis of small molecules with MALDI-MS is complicated by the poor reproducibility of the absolute

intensities of the analyte signals. Reproducibility of the signal intensities depends to a large extent on the type of matrix used (Sleno and Volmer, 2005a), and on the sample preparation (van Kampen et al., 2008a,b). For example, when the widely applied dried-droplet protocol is used, CHCA forms small round crystals that are relatively evenly deposited throughout the spot, whereas 2,5-DHB tends to form different types of crystals with large needle-like crystals in the outer rim and smaller denser crystals in the center of the spot. As a rule of thumb, better reproducibilities are obtained when sample/matrix crystals have a homogeneous appearance and when they evenly cover the spot. The reproducibilities of the absolute signal intensities of quinidine and danofloxacin were compared with the three commonly employed matrices CHCA, 2,5-DHB, and SA that were spotted according to the dried-droplet protocol (Sleno and Volmer, 2005a). The use of CHCA resulted in acceptable precisions of 15% CV and less, whereas the precisions ranged between 23% and 41% CV for SA and from 42% to 90% for 2,5-DHB. Better precisions were obtained when higher matrix concentrations were used, because of a better coverage of the spot with crystals. It has also been noted that strong analyte signals are observed in certain locations of the crystals (the so-called sweet spots), whereas less intense or even no signals are obtained in other parts of the crystals. The commonly held notion to explain this phenomenon is that the analyte is inhomogeneously distributed in the crystals (Hillenkamp & Karas, 2007). Matrix and analytes tend to partition during slow crystallization of the sample when solubilities of the components in the sample are not matched (Hensel, King, & Owens, 1997). Segregation is of particular concern when solvent mixtures are used, and one of the solvent components evaporates more easily than the other (Chen & Guo, 1997; Hoteling, Mourey, & Owens, 2005). The use of azeotropic mixtures can avoid fractional precipitation of the analytes during sample evaporation (Hoteling, Mourey, & Owens, 2005). The sweet spot phenomenon has also been attributed to a different ionization state of the analyte in different parts of the crystals, and to a heterogeneous orientation of the crystals relative to the mass spectrometer axis (Hillenkamp & Karas, 2007). Matching analyte and matrix in terms of relative polarity is also important for homogeneous incorporation of the analyte in the matrix crystals (Hoteling et al., 2004).

B. Increasing Homogeneous Sample/Matrix Crystallization

Practice has shown that precision can be improved by forcing a homogeneous distribution of the analytes in the crystals, and/or by forcing homogeneous sample/matrix crystals that are evenly distributed throughout the spot. Improved homogeneity of matrix crystals can be obtained when matrix surfaces are prepared by fast evaporation (Vorm, Roepstorff, & Mann, 1994). First, a thin matrix layer of microcrystals is obtained by spotting a matrix solution that contains a high percentage of volatile organic solvent; for example, acetone. Samples dissolved in any solution that does not re-dissolve the matrix are deposited on top of the thin layer. This approach is widely used for cinnamic acid-derived matrices such a CHCA and SA, which do not dissolve or poorly dissolve in solutions with high water content. For water-soluble matrices such as 2,5-DHB, this approach is complicated. Mixtures of analyte and matrix in a solution that contains a high percentage of volatile organic solvent can also be used to create sample/matrix crystals with a more homogeneous appearance that are more evenly distributed throughout the spot.

In the seed-layer approach (Westman, Nilsson, & Ekman, 1998; Onnerfjord et al., 1999), a diluted matrix solution that contains a high percentage of organic solvent is spotted on the target plate and is allowed to dry. An aqueous solution of sample and matrix is deposited on the dried matrix crystals, which acts as seeds for crystallization, to produce homogeneous sample surfaces.

In the crushed-crystal method (Westman et al., 1994; Xiang, Beavis, & Ens, 1994), matrix solution is allowed to crystallize on the target plate in a standard dried-droplet protocol; crystals are crushed, and the sample/matrix mixture is spotted onto the crushed crystals.

We have shown an improved precision for the analysis of HIV-1 protease inhibitors with 2,5-DHB as matrix by adding a small amount of dimethylsulfoxide to the sample/matrix mixture (van Kampen et al., 2008a). Although the deposited sample/matrix solutions dry at a much slower rate, homogeneous sample surfaces are obtained. Additives such as fucose (Gusev et al., 1995) also improve the homogeneity of the crystal structures.

Hydrophobic target plates can also be used to improve the homogeneous sample/matrix crystallization as well as to achieve a concentration of the sample/matrix crystals onto a smaller area (Schuerenberg et al., 2000; Owen et al., 2003; van Kampen et al., 2008b). In the AnchorChip technology (Schuerenberg et al., 2000), hydrophilic anchors of 200–800 μm in diameter are placed onto a hydrophobic surface to allow more control over the size of the area onto which the sample/matrix crystals are deposited compared to hydrophobic target plates without anchors. Hydrophobic target plates can be prepared relatively easily by applying hydrophobic coating such as Scotch Gard (Owen et al., 2003) or fluoropolymers (van Kampen et al., 2008b) onto normal stainless steel target plates. For the novel matrix 7-hydroxy-4-(trifluoromethyl)coumarin (HFMC), we have shown that precision improves when sample/matrix mixtures with a high percentage organic solvent are deposited onto a target plate that is coated with a strongly hydrophobic fluoropolymer (van Kampen et al., 2008b). Furthermore, improved LOQs and accuracies were obtained, and the sample/matrix crystals were concentrated onto a small area, even with sample volumes $\leq$10 μL.

Homogeneous thin microcrystal layers can be obtained by a thermal vapor deposition of matrix (Kim, Shin, & Yoo, 1998; Hankin, Barkley, & Murphy, 2007; Dekker et al., 2009). Dekker et al. (2009) use an in-house built sublimation/deposition device to deposit 2,5-DHB onto cytocentrifuged cells that were cultured in the presence of HIV protease inhibitors (see Fig. 3). Pre-spotted target plates that contain, for example, CHCA spots, can also be prepared in this way.

Sophisticated spotting devices such as electrospray deposition instruments (Hensel, King, & Owens, 1997; Wei et al., 2004; Wagner, Varesio, & Hopfgartner, 2008) or piezo-electric droplet dispensers (Little et al., 1997; Allmaier, 1998; Onnerfjord et al., 1998) can be used to obtain a highly homogeneous layer of

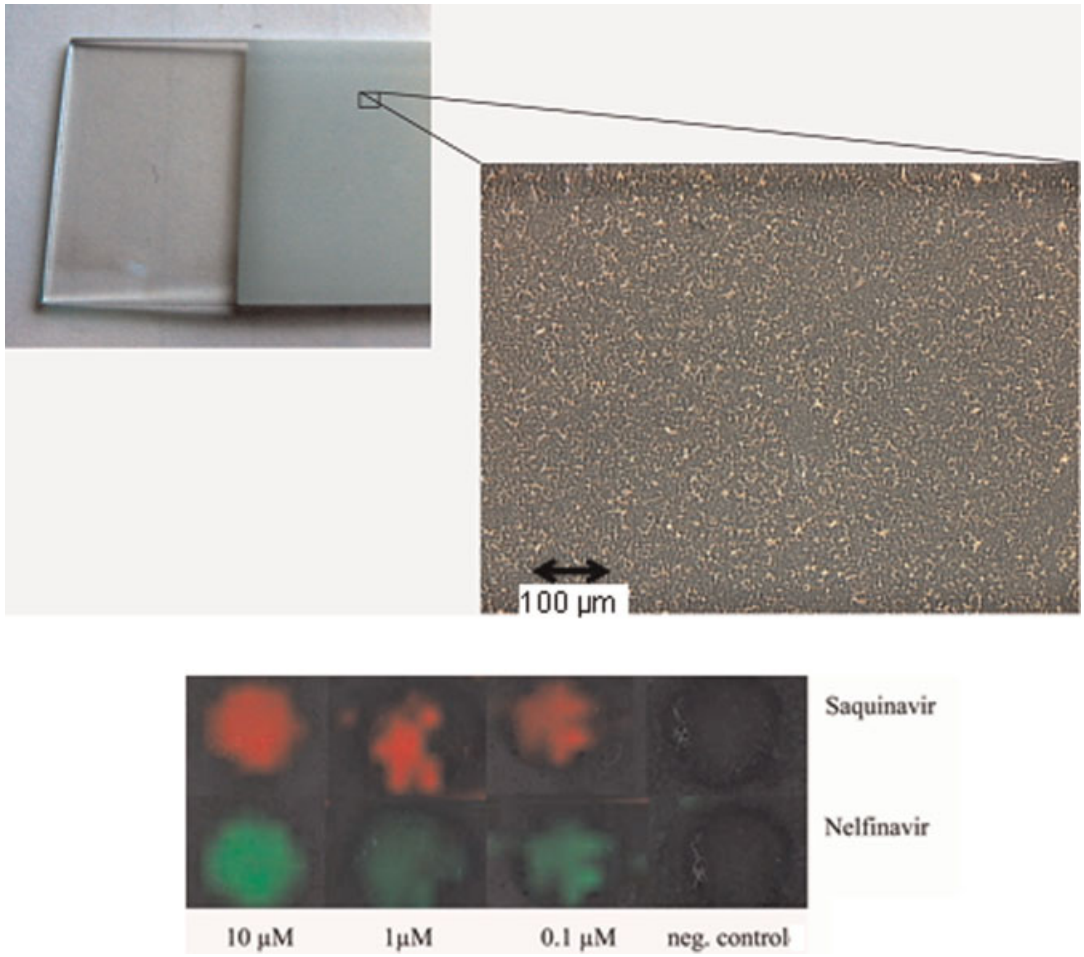

FIGURE 3. Sublimation deposition of matrix. The picture above shows the sublimation/deposition of 2,5-DHB onto a glass slide. The picture below shows the application of this technique to the analysis of cytocentrifuged Mono Mac 6 cells that were cultured in the presence of the HIV protease inhibitors saquinavir and nelfinavir. Color intensities represent the signal intensities of saquinavir (red) and nelfinavir (green) in the cytocentrifuged cells. Reprinted with permission from Dekker et al. (2009), copyright 2009 John Wiley & Sons, Ltd.

co-crystallized sample and matrix to thereby improve the precisions compared to dried-droplet sample preparation. These sophisticated spotting devices might be particularly useful when matrices are used that are known for their heterogeneous crystallization behavior such as 2,5-DHB. Furthermore, these spotting devices might be used to force homogeneous co-crystallization of analyte and internal standard even when segregation occurs under dried-droplet sample-preparation conditions (Sleno & Volmer, 2006).

C. Ionic Liquid Matrices

Armstrong et al. (2001) showed that some room-temperature ionic liquids (RTIL) can be used as effective matrices in UV-MALDI. The RTIL that possess matrix properties are often called ionic liquid matrices (ILM), although ionic matrices or class II RTIL are also used to describe these matrices. RTIL are a class of compounds that consist of ions, have melting points $<100^{\circ}$C and are often liquid at room temperature, and have negligible vapor pressure (Wasserscheid & Keim, 2000). Most ILM form smooth viscous films on the target plate and retain this property under vacuum. ILM can be obtained by mixing a commonly used crystalline MALDI matrix such 2,5-DHB, CHCA, or SA with an equimolar amount of organic base such as tributylamine, pyridine, or 1-methylimidazole (Zabet-Moghaddam, Heinzle, & Tholey, 2004). As a rule of thumb, the preference of the crystalline matrix for a certain class of analytes is retained when these matrices are converted into ILM. An interesting property of several ILM for the analysis of small molecules is that the amount and intensity of matrix-derived peaks is decreased (Tholey & Heinzle, 2006). The greatest advantage of ILM is that these matrices allow for homogeneous sample preparation, which significantly improves the reproducibility of MALDI. ILM have been tested for the analysis of various small molecules (Zabet-Moghaddam, Heinzle, & Tholey, 2004), metabolome analysis (Vaidyanathan, Gaskell, & Goodacre, 2006), oligodeoxynucleotides (Li & Gross, 2004), oligosaccharides (Mank, Stahl, &

Boehm, 2004), peptides, and proteins (Li & Gross, 2004; Mank, Stahl, & Boehm, 2004), and even for tissue imaging (Lemaire et al., 2006). Zabet-Moghaddam, Heinzle, and Tholey (2004) reported that peak intensities of small molecules in solid matrices varied by more than 60%, whereas variations of less than 10% were observed for their ionic liquid forms. The correlation coefficients for the calibration curves also improved when ILM were used. Other properties of ILM compared to their solid counterparts are that slightly increased laser fluences are needed for ionization, and that ILM favor formation of sodium and potassium adducts (Mank, Stahl, & Boehm, 2004; Zabet-Moghaddam, Heinzle, & Tholey, 2004); those properties can be exploited for analytes with poor protonation (Gouw et al., 2002; van Kampen et al., 2006, 2008a, 2009b). For further information on ILM, see the review of Tholey and Heinzle (2006).

D. Internal Standards

The use of an internal standard is indispensable for the robust and reproducible quantitative analysis of small molecules with MALDI-MS. Ideally, the internal standard compensates for all variations in analyte recovery during the sample-preparation process and yields identical mass spectrometric behavior as the analyte. In MALDI-MS, the internal standard should compensate for crystallization irregularities and for desorption and gas-phase effects. In particular, the crystallization irregularities might present a problem in quantitative MALDI-MS. Matrix, analyte, and internal standard tend to partition during the crystallization process (Chen & Guo, 1997; Hensel, King, & Owens, 1997; Hoteling et al., 2004; Hoteling, Mourey, & Owens, 2005; Sleno & Volmer, 2006). In mixed solvents such as acetonitrile/water and methanol/water where one component evaporates faster than another, such segregation effects might be of particular concern. The use of azeotropic mixtures avoids segregation of components during crystallization. The stable isotope-labeled version of an analyte is regarded as the ideal internal standard, because solvent, desorption, and gas-phase properties are nearly identical to that of the analyte (Wilkinson et al., 1997; Kang, Tholey, & Heinzle, 2001). The stable isotope-labeled internal standard and analyte are incorporated into the matrix crystals in the same extent and in the same locations, and therefore heterogeneous sample/matrix crystallization might not cause a detrimental effect on precision and accuracy. However, such stable isotope-labeled internal standards are expensive and might not be commercially available, and, in practice, other pharmaceutical compounds are frequently used as internal standards. Precise and accurate quantification can be obtained using ''look-alike'' pharmaceutical compounds as internal standards. Sleno and Volmer (2006) investigated the influence of solution-phase ionization equilibria and hydrophobicity on the relative response of analyte and internal standard. The predicted apparent octanol/water partition coefficient D was a good parameter to search for an appropriate internal standard; precise and accurate quantitation was obtained only when analyte and internal standard had a similar log D (see Fig. 4). Furthermore, when analyte and internal standard had matching log D's and thus a good co-crystallization, precise and accurate quantification could be obtained even when the analyte and internal standard were not homogeneously distributed throughout the whole crystallized sample.

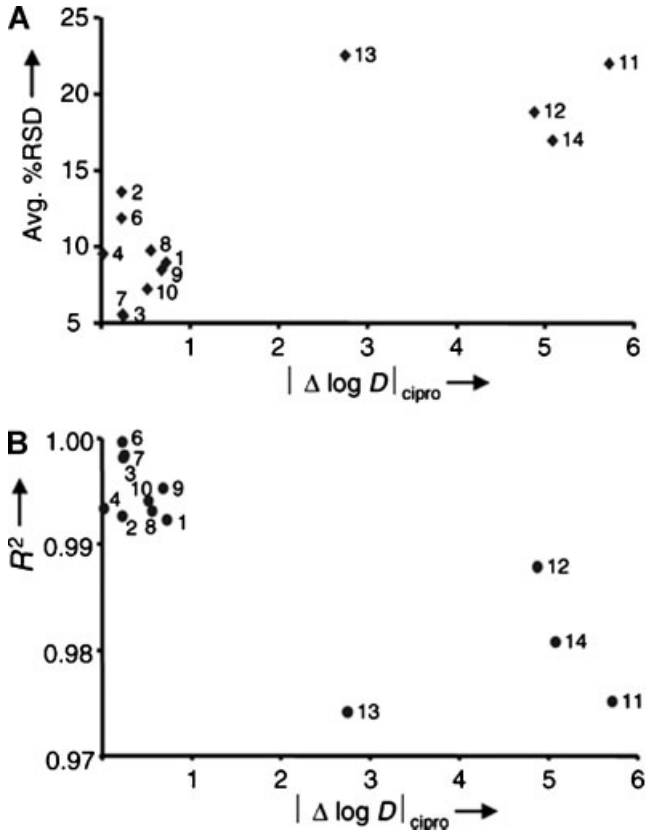

FIGURE 4. Octanol/water partition coefficients of analyte and internal standards. Quantitation of ciprofloxacin with 13 different 4-quinolones as internal standard. The y-axis shows % RSD or r^2 for quantitation of ciprofloxacin. The x-axis shows the difference in log D between ciprofloxacin and the internal standard. CHCA dissolved in acetonitrile/water (1:1; v/v) and 0.2% TFA was used as matrix. Log D values were predicted for pH 2.0. Reprinted with permission from Sleno and Volmer (2006), copyright 2009 John Wiley & Sons, Ltd.

E. Averaging

A common method to improve reproducibility is to average a large number of individual data points. In this respect, firing the laser at a higher frequency is advantageous to obtain a good precision without compromising sample analysis times. Comparison between laser firing at 20 and 1,000 Hz for analysis of clonazepam and nordiazepam showed that the higher laser frequency improved the within spot and spot-to-spot precisions by at least a factor of 5 (Hatsis et al., 2003).

F. Algorithms for Data Collection and Analysis

Nicola et al. (1998) developed a correlative analysis algorithm for automated data collection to improve the quality of the average mass spectrum by compensating for shifts along the mass axis of the single-shot MALDI-TOF spectra. This method resulted in an

improved mass resolution (3×), better reproducibility of signal intensities (4×), and better quantitative accuracy (1.3×). Horak, Werther, and Schmid (2001) compared data acquisition at constant laser power with data acquisition at constant ion abundance for quantitative analysis of chlormequat. Data acquisition at constant ion abundance resulted in a better sample-to-sample reproducibility compared to data acquisition at a constant laser power. We tested the effect of baseline subtraction, peak parameter (intensity, S/N, or area), and analyte signal (monoisotope vs. all isotopes) on the precision of lopinavir and ritonavir analyzed by MALDI-TOF and found that the best % CVs were obtained when the monoisotopic peak area without baseline subtraction was used (van Kampen et al., 2006).

V. PRACTICAL ASPECTS OF SMALL-MOLECULE ANALYSIS WITH MALDI

A. Protonation Versus Cationization

For biopolymers such as proteins, peptides, and nucleic acids, a (de)protonation strategy is usually pursued, and the formation of salt adducts is regarded as disadvantageous. It is well known that additives can significantly decrease these salt adduct formations. We have used DAHC as an additive in the binary matrix nicotinic acid/anthranilic acid (55/45/45 mM, respectively) to decrease the amount of salt adducts for the analysis of (deoxy)nucleotide triphosphates and the triphosphosphorylated form of the nucleoside reverse transcriptase inhibitor zidovudine in negative-ion MALDI-TOF experiments (van Kampen et al., 2004). An advantageous additional effect of DAHC and other additives is that they result in a more homogeneous crystallization of matrix and analyte to thereby improve the precision.

Cationization strategies are, however, preferred for analytes that lack basic sites such as oligosaccharides and synthetic polymers. We have successfully pursued a cationization strategy to quantify drugs with MALDI-TOF (van Kampen et al., 2006, 2007), MALDI-FTICTR (van Kampen et al., 2008a), and MALDI-triple quadrupole MS (van Kampen et al., 2008b, 2009b). For the high MW porphyrin matrix F20TPP, we tested which alkali metal, that is, Li, Na, K, Rb, or Cs, produced the strongest signal for cationized drugs (van Kampen et al., 2006), and we found that analytes were most efficiently ionized by Li^+ attachment. This result was surprising, because analysis of mixtures of the F20TPP matrix with equimolar amounts of LiI, NaI, KI, RbI, and CsI revealed that hardly any free Li^+ is detected. The intensities of the free alkali-metal ions showed the distribution of $Li^+(=0) < Na^+ < K^+ < Rb^+ < Cs^+$, that result can be explained by the inverse relationship of the alkali iodide lattice energies and size of the alkali metal. Thus, with the same halide as counterion, the larger the alkali metal the lower the lattice energy, to liberate more alkali-metal ions during the desorption/ionization process. This distribution is also observed when 2,5-DHB is used as the matrix. On the other hand, studies with LMW poly(ethyleneglycols) (PEGs) showed that the intrinsic affinity of analyte for the alkali-metal ions follows the order of $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. Thus, the intrinsic metal ion affinity appears to be governed primarily by the charge density of the cation. In practice, the quantitative distribution of PEG ionized with alkali metals can be rationalized on the basis of a trade-off between these two opposing effects; namely, the gas-phase availability of the alkali cation ($Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$) and the intrinsic alkali affinity of PEG ($Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$) leads to an observed optimum affinity for K^+ (van Kampen et al., 2008a, 2009a). The optimum affinity for K^+ is indeed the case when 2,5-DHB is used as the matrix. With F20TPP, the most abundant signals are observed for PEG cationized by Li^+, followed by Na^+, hardly any kaliated signals are observed, and virtually no adducts are formed with rubidium or cesium (see Fig. 5). Furthermore, the binding efficiencies of Li^+ towards LMW PEGs strongly depend on the number of sites where the Li^+ ion can interact (Bogan & Agnes, 2002; van Kampen et al., 2009a). The intensity order of F20TPP itself cationized by various alkali metals is very similar to that of the LMW PEGs; that is, $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. We propose that, for porphyrins, the cationized matrix might well be the cation donator to the analytes, whereas analyte cationization with other matrices seems to occur predominantly through attachment of free cations in the gas phase (Zhang & Zenobi, 2004). *Ab initio* calculations showed that Li^+ is attached to the porphyrin cavity and not to the F atoms of the C_6F_5 groups. Equilibrium structures for porphyrin + Li^+ showed that the Li^+ is situated on, but not in, the porphyrin cavity, and that the whole porphyrin chain becomes bent. The observation that the lithium ion is not in, but outside, the cavity means that the lithium ion is exposed and is therefore accessible for transfer to analyte molecules. The larger the cation, the more exposed the cation is (and the lesser the porphyrin skeleton will be bent); however, at the same time, the cation affinities become less (see Fig. 6). Our experiments showed that only lithium ions produce intense signals for attachment to F20TPP, and that such F20TPP + Li^+ ions might act as cation donators. The preference for Li^+ attachment to matrix and analyte was also found for other porphyrin matrices.

Hoberg et al. (1998) showed that, when an alkali-metal cationization strategy is pursued with alkalihalide salts, a halide counter anion should be chosen that results in the lowest lattice energy of the alkalihalide salt. For example, when cationization with sodium is pursued, more abundant sodiated signals for the analyte are observed when sodium iodide is used as the cationizing agent compared to sodium chloride. The proton affinity of porphyrin is large (245 kcal/mol), and thus many analytes cannot be charged by protonation with this matrix. Indeed, for various drugs we found no protonated molecules with F20TPP. We have, however, found evidence for protonated fragments of protease inhibitors (van Kampen, unpublished experiments), and it might be that, for protonation, F20TPP should be regarded as a hot matrix that can result in prompt in-source fragmentation of protonated analytes. On the other hand, Kosanam et al. (2007) successfully applied the F20TPP matrix for the quantitative analysis of doping agents in urine with MS/MS on the protonated analytes in MALDI-ion trap experiments.

Preference for certain cations also depends on the nature of the analyte. For example, Burgers and co-workers showed that tryptic peptides that contain the calcium-binding domain of calcium-binding proteins retain their preference for calcium in MALDI experiments, and that intense signals are observed for

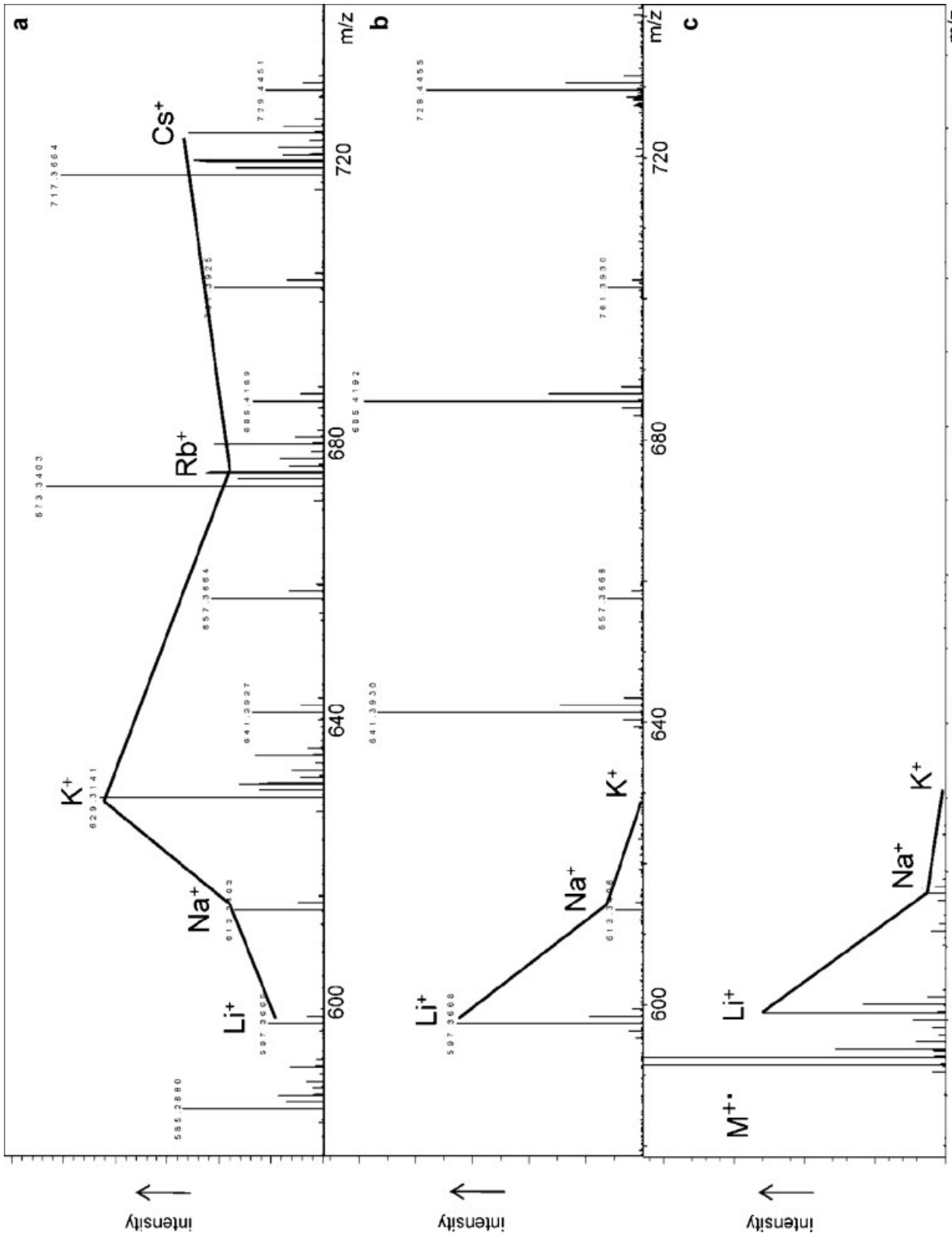

FIGURE 5. Porphyrin matrix and alkali cation distribution of analyte. Alkali-metal distribution of PEG with an equimolar mixture of LiI, NaI, KI, RbI, and CsI in combination with the matrix 2,5-DHB (**a**) and F20TPP (**b**). Alkali metal of the matrix F20TPP itself (**c**). Reprinted with permission from van Kampen et al. (2009a), copyright 2009 John Wiley & Sons, Ltd.

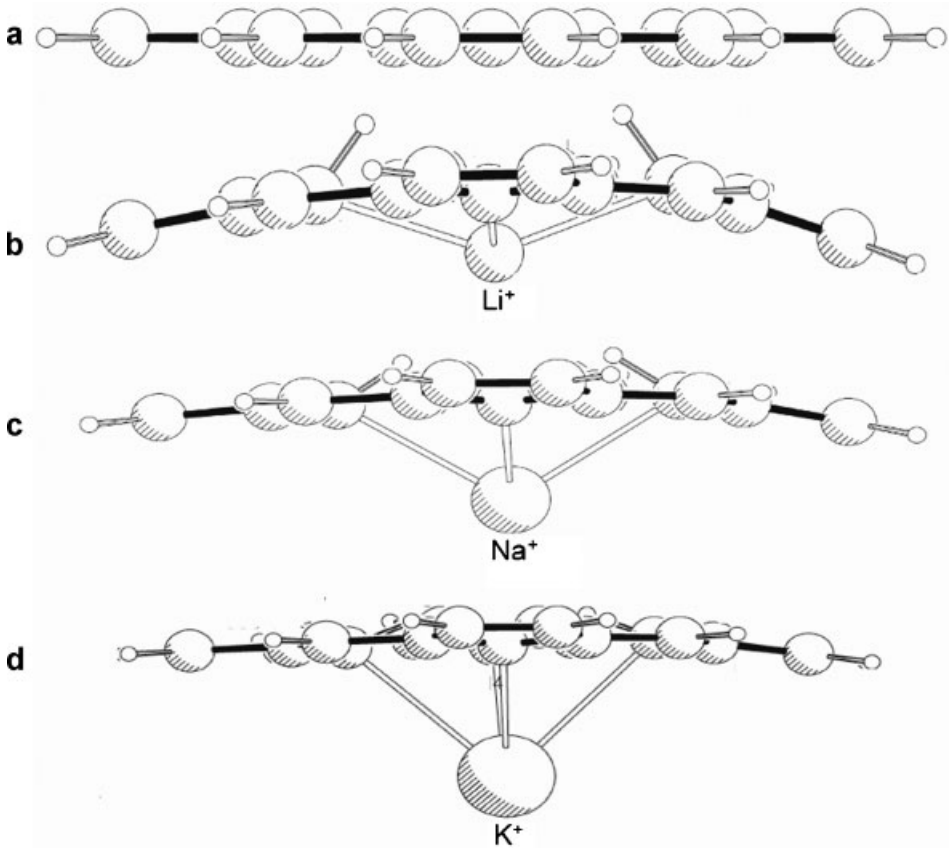

FIGURE 6. Interaction of porphyrin with alkali metals. Side views of porphyrin + H^+ (**a**), porphyrin + Li^+ (**b**), porphyrin + Na^+ (**c**), and porphyrin + K^+ (**d**). Reprinted with permission from van Kampen et al. (2009a), copyright 2009 John Wiley & Sons, Ltd.

the calciated peptides (Jobst et al., 2008). SRM experiments on a MALDI-triple quadrupole MS showed that protonated precursor molecules produce product ions of higher intensity than cationized precursor ions (van Kampen et al., 2008a). Furthermore, for analytes cationized with the larger alkali cations (K^+, Rb^+, Cs^+), MS/MS results in loss of the analyte as a neutral. This fragmentation pattern is due to the low affinity of the larger cation towards the analyte (Francis et al., 2005). With CHCA as matrix to quantify five HIV protease inhibitors on a MALDI-triple quadrupole MS, we found weak signals for the protonated molecules of lopinavir and ritonavir; SRMs on the sodiated forms of lopinavir and ritonavir, however, resulted in sensitive, precise, and accurate quantification (van Kampen et al., 2009b). An interesting matrix for cationization of various small molecules is lead pencil. Black et al. showed that pencil lead, which contains graphite, clay, and ''gliding'' agents such as wax, acts as a matrix for MALDI-MS (Black et al., 2006; Langley, Herniman, & Townell, 2007). With this matrix, the success rate ($S/N > 5$; ~10 ng spotted) was 88% for a library of 50 small molecules that contained tripeptides, steroids, drugs, herbicides, dyes, and PEGs. Examples of drugs included in the library are haloperidol, carbamazepine, tamoxifen and 4-hydroxy tamoxifen, erythromycin, cefuroxime, and mebendazole. In particular, intense signals for the sodiated or kaliated analytes were observed. Indeed, with lead pencil as matrix to analyze the HIV protease inhibitor saquinavir in MALDI-TOF and MALDI-FTICR experiments, strong signals were found for the sodium and potassium adducts but not for protonated saquinavir (van Kampen, unpublished experiments). Those data contrast sharply with matrices such as 2,5-DHB and CHCA, which yield intense signals for the protonated molecule (Wagner, Varesio, & Hopfgartner, 2008; van Kampen et al., 2009b). Adding either a sodium salt or a potassium salt to the sample enhances analyte cationization and thereby improves the sensitivity (van Kampen, unpublished experiments). Charcoal behaves in a similar way as pencil lead, although this matrix needs to be applied on to a target plate with a rough surface; for example, a groundsteel target plate (Burgers, unpublished experiments).

B. MS Versus MS/MS

For quantitative analysis of drugs in the MS mode, high-resolution and high mass accuracy are advantageous because higher mass accuracy results in a higher certainty that the peak in the mass spectrum is actually the drug of interest, and high resolution increases the selectivity to discriminate between the analyte and isobaric matrix interferences. An additional advantage of high mass accuracy MS measurements over a targeted MS/MS approach for quantitative analysis of drugs is

that the accurate masses of other peaks than the drug itself present in the mass spectrum could be used to establish whether these peaks are (unexpected) drug metabolites or not. We propose that MALDI instruments such as TOF, Orbitrap, and FTICR are suitable for discovery-type applications. Neutral-loss scans and precursor-ion scans on a triple quadrupole instrument can also be used to search for drug metabolites. For targeted quantitative analysis of drugs, we propose an MS/MS approach. Using this approach, conventional small-molecule matrices can be used without any problems that are associated with matrix-derived interfering signals. Furthermore, MS/MS approaches normally result in higher sensitivities. In this respect, the triple quadrupole mass spectrometer would be the instrument of choice because such an instrument allows for SRM with a nearly 100% duty cycle.

C. Preparation of Biological Samples

Relatively simple, fast, and easy-to-automate procedures that allow batch preparation of biological samples are needed to truly benefit from the high-throughput measurements of MALDI-MS. Sample-preparation procedures in 96- or 384-well plates are highly suited for MALDI, because these formats are widely used for MALDI target plates and allow a relatively easy spotting of the processed samples with a pipetting robot (Wu et al., 1997; van Kampen et al., 2006; Wagner, Varesio, & Hopfgartner, 2008).

Preparation of samples with SPE, LLE, and PP has been tested for quantitative analysis of small molecules by MALDI-MS. The processed samples are normally dried after these procedures and are re-constituted in a smaller volume of an appropriate solvent or matrix solution. This drying step is frequently needed to maintain the required sensitivity because sample volumes of only 0.5–1 μL are deposited onto the target plate for measurement. We have shown that SPE-processed samples can be re-constituted in volumes as low as 10 μL for sensitive, precise, and accurate quantitation of HIV protease inhibitors in cell lysates (van Kampen et al., 2008a,b). Gobey et al. (2005) showed that the drying step can be omitted for SPE-processed plasma by eluting the samples from the SPE columns with 25 μL matrix solution. Wagner, Varesio, and Hopfgartner (2008) used LLE to process plasma samples from HIV-infected patients who were treated with the protease inhibitor saquinavir. When PP is used, however, the drying step has a detrimental effect on the crystallization of the sample with a concordant severe loss of sensitivity (Gobey et al., 2005; Kovarik et al., 2007; Wagner, Varesio, & Hopfgartner, 2008). For quantitative analysis of HIV protease inhibitors in plasma of HIV-infected children, we found that good crystallization and high sensitivity can be obtained for protein-precipitated samples when the drying step is omitted (van Kampen et al., 2009b). Ten microliters of plasma was ''diluted'' in 90 μL methanol that contained the internal standard, the supernatant was diluted in matrix solution (1:2, v/v), and 0.75 μL matrix/sample solution was deposited onto a target plate; that is, the plasma sample was diluted 30 times in an organic solvent. Detrimental effects on crystallization were observed only when the solvent that was used for the PP consisted of a high percentage of water, or when lower dilution was used.

Analytes can also be captured from biological samples with target plates with functionalized surfaces (Cohen, Go, & Siuzdak, 2007). Functionalized magnetic nanoparticles and water-soluble fullerene derivatives, which serve also as a MALDI matrix, have been used to capture analytes from complex biological samples (Shiea et al., 2003; Lin et al., 2007).

VI. BIOMEDICAL APPLICATIONS OF MALDI

A. Quantitative Analysis of Drugs

Although many studies have shown promising results for MALDI-MS quantitative analysis of small molecules, real-life applications of MALDI-MS for the analysis of drugs in biological samples are still limited. Below, we discuss some of the studies that have shown successful application for MALDI-MS for quantitative analysis of drugs.

Matrix-assisted laser desorption/ionization (MALDI)-triple quadrupole MS has been used for the analysis of several spirolide toxins in phytoplankton (Sleno and Volmer, 2005b). Samples were extracted in methanol, and LC fractionated. For some samples, an additional SPE procedure was used. Cross-validation showed good agreement for the quantitation obtained with MALDI-MS and ESI-MS. Furthermore, MALDI-MS precursor-ion scans and neutral-loss scans were successfully applied to discover unknown spirolide analogs in the phytoplankton samples.

Gobey et al. (2005) have applied MALDI-triple quadrupole MS to the rapid analysis of human liver microsome half-lives of 53 pharmaceutical compounds. Comparison with ESI-MS showed good agreement among the assays.

Rideout, Bustamante, and Siuzdak (1993) applied MALDI-TOF MS for quantitative analysis of the cationic drug tetraphenylphosphonium (TPP) in carcinoma cell lines. Methyltriphenylphosphonium was used as the internal standard. The cells incubated with the drug were prepared by simple lysis with a freeze–thaw cycle. Subpicomole amounts of the drug were quantified, and scintillation counting showed good agreement with the MALDI-TOF assay. Furthermore, the assay was used to monitor the formation of hydrazones from aldehydes and hydrazine derivates inside cells. MALDI-TOF MS has also been used to study the uptake of TPP and other phosphonium cations in cell lines (Cheng, Winant, & Gambhir, 2005) and the uptake of cell-penetrating peptides (Burlina et al., 2005).

We used MALDI-TOF and MALDI-FTICR MS to image HIV protease inhibitors in MonoMac-6 cells cultured in the presence of 0.1–10 μM saquinavir and nelfinavir (Dekker et al., 2009). Cells (2×10^5) were cytocentrifuged on electrically conductive glass slides, and matrix was applied with an in-house build sublimation/deposition device. To obtain good sensitivities, the deposited matrix was re-crystallized with water vapor; this re-crystallization procedure did not result in loss of spatial information.

Volmer et al. (2007) compared MALDI-triple quadrupole MS and LC–MS for the plasma pharmacokinetic analysis of two drug candidates that were administered to rats. Chemical analogs were used as internal standards, and plasma samples (125 μL)

were prepared with SPE. The LOQs were 536 nM (osteoporosis candidate drug) and 426 nM (asthma candidate drug) for the LC–MS assay and the MALDI-MS assay. There was excellent agreement between the pharmacokinetic parameters obtained with MALDI-MS and with LC–MS.

Muddiman et al. (1994) applied MALDI-TOF and secondary ion mass spectrometry (SIMS)-TOF to quantify cyclosporine A in the whole blood of transplant patients. Cyclosporine D was used as internal standard, and whole blood samples were prepared with LLE. The LOQ was 19 nM for SIMS-TOF and 33 nM for MALDI-TOF. It was also demonstrated that these techniques can provide information on cyclosporine metabolites. Wu et al. (1997) used an automated MALDI-TOF platform to quantify this drug. Tacrolimus and its hepatic metabolites were quantified with MALDI-TOF and SIMS-TOF MS (Gusev et al., 1996).

Wagner, Varesio, and Hopfgartner (2008) used MALDI-triple quadrupole MS to quantify the HIV protease inhibitor saquinavir in plasma from HIV-infected patients. LLE was used to prepare the plasma samples (250 μL), and deuterated saquinavir was used as the internal standard. The LOQ was 7.5 nM. Cross-validation between MALDI-MS and LC–MS of clinical samples showed an excellent agreement between the assays. To investigate the effect of co-medication, which is frequently encountered in clinical applications, on the performance of the assay, eight antiretroviral drugs were spiked to human plasma that contained saquinavir. Selectivity and accuracy were not compromised by the presence of these other antiretroviral drugs. Further experiments showed that reserpine could also be used as an appropriate internal standard.

Notari et al. (2008) used MALDI-TOF/TOF MS to quantify the antiretroviral drugs abacavir, stavudine, didanosine, efavirenz, nevirapine, and amprenavir in the plasma of HIV-infected patients. 4-Hydroxybenzoic acid was used as the matrix. A plasma sample (600 μL) was used, and sample preparation consisted of an SPE. The limits of quantification were 10 nM. Cross-validation between MALDI-TOF/TOF and high-pressure liquid chromatography with UV detection (HPLC-UV) showed an excellent agreement.

Antiretroviral drugs have been quantified with MALDI-TOF (van Kampen et al., 2006, 2007), MALDI-FTICR (van Kampen et al., 2008a), and MALDI-triple quadrupole MS (van Kampen et al., 2008b, 2009b). A MALDI-triple quadrupole yielded the best applicability for clinical studies and was used to investigate the plasma and intracellular pharmacokinetics of the HIV protease inhibitors lopinavir and ritonavir in HIV-infected children. Plasma samples were processed with PP with methanol, and cell samples were processed with a methanol extraction followed by SPE. The methanol-extraction step simultaneously inactivated HIV, and further sample handling after this step can be performed under less-stringent biosafety conditions (van Kampen et al., 2007). The assays were specifically developed for application in children, and care was taken that quantification could be performed on a small amount of patient sample. Accurate and precise quantification was obtained from only 10 μL plasma or one million peripheral blood mononuclear cells (PBMC; ∼0.4 μL intracellular volume). Furthermore, an assay was developed for multiplexed quantitative analysis of saquinavir, nelfinavir, and indinavir with methotrexate as the internal standard. Nelfinavir was used as the internal standard to quantify lopinavir and ritonavir in plasma and in cells. The LOQs obtained in plasma were 167 nM (lopinavir), 16 nM (indinavir, nelfinavir), 15 nM (ritonavir), and 3.2 nM (saquinavir). The LOQs in PBMC were 834 nM for lopinavir and 73 nM for ritonavir. To investigate the effect of co-medication on the assays, a mixture of 10 drugs was added to plasma samples from an HIV-infected child treated with Kaletra (lopinavir/ritonavir). The added drugs did not affect precision or accuracy for lopinavir and ritonavir as shown by an analysis of the same samples without the drug mixture, and a cross-validation with HPLC-UV.

B. Metabolomics

The suitability of MALDI-MS for metabolome analysis has been investigated (Vaidyanathan & Goodacre, 2007; Vaidyanathan et al., 2007). These studies aimed to simultaneously identify and quantify a cocktail of metabolites with a few internal standards. It was shown that MALDI-MS can be used for the simultaneous analysis of a cocktail of metabolites. However, analyte suppression was observed for some metabolites when the relative portion of one analyte dominated the others in the mixture. Furthermore, the isomeric interferences encountered in metabolomics applications show the need for MS/MS and/or appropriate sample-preparation procedures. For example, ATP and dGTP have the same elemental composition but can be separated from each other in MALDI post-source decay experiments (van Kampen et al., 2004). Offline spotting of LC fractions on target plates is frequently used for MALDI in proteomics to increase the number of identified peptides. Such approaches might also proof useful for MALDI-MS metabolomics. LC–MALDI and LC–ESI of the same set of samples showed that only one-third of all identified peptides were found by both techniques, and that the techniques can thus be regarded as complementary (Rompp et al., 2007; Stoop et al., 2008). Nordstrom et al. (2008) showed that a multiple ionization strategy consisting of ESI, atmospheric pressure chemical ionization, and DIOS MS significantly increased the number of ions detected in metabolome analysis. Another approach to increase the success rate in metabolome analysis, which is also suitable for pharmaceutical compounds, is charge derivatization of small molecules or derivatization of analyte molecules with a matrix (Brombacher, Owen, & Volmer, 2003; Lee, Chen, & Gebler, 2004).

C. Monitoring the Conversion of Small-Molecule Substrates by Enzymes

An interesting application of MALDI-MS is to monitor enzymatic reactions with small-molecule substrates. In this approach, substrate and product are simultaneously detected, and the substrate-to-product ratio is calculated to quantitatively monitor the enzymatic reactions without an internal standard (Whittal et al., 1995; Kang, Tholey, & Heinzle, 2000; Shen et al., 2004; Tholey, Zabet-Moghaddam, & Heinzle, 2006; Rathore et al., 2008). For example, MALDI-triple quadrupole MS was also used to screen for kinase inhibitors of cyclic AMP-dependent protein kinase (Rathore et al., 2008). The substrate

in this assay was the peptide kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), which is phosphorylated by the protein kinase.

VII. FUTURE DIRECTIONS

Currently, there is still no comprehensive model that accurately predicts the MALDI mass spectra in a qualitative and quantitative way given a certain sample, matrix, sample preparation, and instrument, even though much progress has been made in this area (Knochenmuss, 2003, 2006; Hillenkamp & Karas, 2007). We propose that a better mechanistic understanding of the relative response of analyte and internal standard in biological samples greatly benefits the application of MALDI for targeted quantitation of small molecules, and for metabolomics applications. Such an understanding could be used to correct for differential-suppression effects (i.e., unequal suppression of analyte and internal standard) and improves MALDI quantification and reliability. The apparent octanol/water partition coefficients have already been shown to be useful to predict which internal standard should be used for precise and accurate quantitation (Sleno & Volmer, 2006). A better understanding of the MALDI mechanism can also be used to discover or even synthesize new matrices for MALDI. Sometimes, we test for matrix properties of molecules when they have favorable UV-light absorption profiles; that process led to the discovery of the novel matrix HFMC (van Kampen et al., 2008b). *Ab initio* calculations were used to create new hypotheses of how matrices act in MALDI such as in the case of porphyrin matrices (van Kampen et al., 2009a). A breakthrough in finding matrices for MALDI was the rational design of the new matrix 4-chloro-α-cyanocinnamic acid (Jaskolla, Lehmann, & Karas, 2008). This matrix showed a much more uniform response to analytes of different basicities than the commonly used CHCA. This approach might also be used to produce new matrices for improved analysis of small molecule or to produce matrices that work particularly well for a certain interesting class of molecules. The position of MALDI as an analytical tool for small-molecule analysis will be strengthened by further improvements of matrix-free approaches (Cohen, Go, & Siuzdak, 2007; Najam-ul-Haq et al., 2007; Petterson, 2007).

Real-life applications of MALDI for small-molecule analysis in biomedical research, diagnostics, and clinical medicine are still limited. We expect that MALDI will be applied in particular when large numbers of samples must be measured (e.g., in epidemiological studies), or when fast results are needed, such as in diagnosis of infectious diseases. Small-molecule markers and conversions of small molecules by enzymes are routinely used to diagnose pathogens in medical microbiology laboratories, and we expect that MALDI-MS can play an important role in this area. For some drugs, therapeutic drug monitoring is routinely applied in a clinical setting; MALDI can also play a significant role in this area. We propose that MALDI should not be seen as an analytical tool that makes tested-and-proven methods such as LC–MS/MS or HPLC-UV obsolete, but that MALDI should be regarded as an addition to the analytical armory. For example, one MALDI mass spectrometer can be used as a workhorse in the setting of university medical center to answer many research questions in a relatively short time. Other analytical tools can be used to translate the positive results to a clinical setting; for example, the finding that the concentration of a certain drug correlates with clinical outcome. We propose that, at this moment, MALDI is not yet ready for routine use in clinical laboratories, and, first, long-term head-to-head comparison with proven analytical assays must be performed and standard operating procedures must be developed that deal specifically with the nature of MALDI; for example, life time of the laser.

Currently, the use of different types of MALDI mass spectrometers is not fully exploited; not the least, concerted measurements are often hampered by practical problems such as variations in the dimensions of the target plates used by different MS vendors. Studies have shown that samples can be stored on target plates in their matrix co-crystallized form, and that sample consumption during measurements is low. The low sample consumption and storage of samples on target plates allow researchers to combine the specific strengths of mass analyzers like FTICR, TOF, Orbitrap, and triple quadrupole to obtain answer to the research questions. For example, the high-resolution and high mass accuracy of MALDI-FTICR can be used to search for (unexpected) drug metabolites in a selected group of samples; a MALDI-triple quadrupole can be used for a fast, relatively quantitative analysis of the drug metabolites in a large number of samples. Storage of samples on a target plate also allows researchers to send samples to other laboratories that have a particular expertise or a particular type of MALDI mass spectrometer that is not present in the original laboratory.

We expect further improvements in the biomedical application of MALDI-MS for small-molecule analysis by the specific development of techniques for clinical samples that range from biofluids, lysates of tissues, and frozen-tissue sections that can be screened with MALDI-MS for specific small molecules.

VIII. CONCLUDING REMARKS

Matrix-assisted laser desorption/ionization (MALDI) MS offers some intrinsic properties that favor its use for small-molecule analysis such as its high tolerance towards salts and buffers, the rapid analyses, its high absolute sensitivity, the small amount of sample consumed during analysis, and the possibility to store samples on a target plate. For this application, however, approaches should be used that are specifically developed for this application to overcome or circumvent matrix-related noise in the low-mass range and the poor reproducibilities of signal intensities. A variety of such approaches have been developed and allow unambiguous qualitative annotation of small molecules as well as precise and accurate quantitation. These approaches are now ready to be tested in a clinical environment.

IX. ABBREVIATIONS

2,5-DHB	2,5-dihydroxybenzoic acid
9-AA	9-aminoacridine
CHCA	α-cyano-hydroxycinnamic acid
CTAB	cetyltrimethylammonium bromide

DAHC	diammoniumhydrogencitrate
DIOS	desorption ionization on porous silicon
ESI	electrospray ionization
F20TPP	meso-tetrakis(pentafluorophenyl)porphyrin
FTICR	Fourier-transform ion cyclotron resonance
HFMC	7-hydroxy-4-(trifluoromethyl)coumarin
HPLC	high-pressure liquid chromatography
ILM	ionic liquid matrix
LC	liquid chromatography
LDI	laser desorption/ionization
LLE	liquid-liquid extraction
LMW	low molecular weight
Log *D*	coefficient of distribution
LOQ	limit of quantification
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MSE	matrix-suppression effect
MW	molecular weight
PBMC	peripheral blood mononuclear cells
PEG	poly(ethyleneglycol)
PP	protein precipitation
QqTOF	quadrupole time of flight
RTIL	room-temperature ionic liquid
S/N	signal-to-noise ratio
SA	sinapinic acid
SIMS	secondary ion mass spectrometry
SRM	selected reaction monitoring
THAP	trihydroxyacetophenone
TOF	time of flight
TPP	tetraphenylphosphonium

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