MALDI matrices for low molecular weight compounds: an endless story?

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Abstract

Since its introduction in 1980s, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has gained a prominent role in the analysis of high molecular weight biomolecules such as proteins, peptides, oligonucleotides and polysaccharides. Its application to low molecular weight compounds has remained for long time challenging due to the spectral interferences produced by conventional organic matrices in the low *m/z* window. To overcome this problem, specific sample preparation such as analyte/matrix derivatization, addition of dopants or sophisticated deposition technique especially useful for imaging experiments, have been proposed. Alternative approaches based on second generation (rationally designed) organic matrices, ionic liquids and inorganic matrices including metallic nanoparticles, have been the object of intense and continuous research efforts. Definite evidences are now provided that MALDI MS represents a powerful and invaluable analytical tool also for small molecules, including their quantification, thus opening new exciting applications in metabolomics and imaging mass spectrometry. This review is intended to offer a concise critical overview of the most recent achievements about MALDI matrices capable of specifically address the challenging issue of small molecules analysis.

Keywords: Mass spectrometry, low molecular weight compounds, metabolomics, MALDI matrices.

Introduction

Although matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) [1] has revolutionized the analysis of large, nonvolatile and labile molecules (such as proteins, peptides, oligonucleotides and polysaccharides) its application to low-molecular weight compounds (LMWC) (otherwise denoted as "small molecules") has been regarded, for long time, not appropriate or even useless. One of the main reason was certainly represented by the large background generated by matrix related ions in the *m/z* range of interest for small molecules (*vide infra*). A second reason was the concurrent explosive growth of electrospray ionization (ESI) source [2] that, being easily interfaced to liquid chromatography (LC), has generated LC-ESI-MS one of the most versatile and powerful analytical tool presently available. However, specific advantages of MALDI (*e.g.* high tolerance towards contaminants and buffers, uncomplicated spectra due to the almost exclusive generation of single charged ions, typical sub-femtomole sensitivity and high throughput analysis) coupled to the most recent improvements in related instrumentations, has provided strong foundations to reassess the role this technique could play in the LMWC scenario. Indeed, the application of MALDI MS to small molecules is continuously growing driven by the resurgence of interest in metabolomics studies and, more recently, by the overwhelming growth of mass spectrometry imaging (MSI) techniques that make the LMWC scenario actually topical.

The successful application of MALDI MS is strictly dependent on the matrix choice that should meet specific requirements including analyte incorporation into an excess of matrix [3], high absorption at the irradiation laser wavelength (*via* electronic or vibrational excitation), efficient analyte ionization, null or minimal and controllable analyte fragmentation. Commonly used matrices typically feature linear conjugated π systems and/or aromatic rings for photons absorption in the UV region and acid functional groups for

proton transfer to analyte. In general, derivatives of benzoic acid, cinnamic acid, and related aromatic compounds are recognized as elective matrices for proteins and peptides [4], while picolinic and succinic acids have been used as matrices for the MALDI MS analysis of oligonucleotides [5].

Since the 'first generation' of MALDI matrices (mostly selected on largely empirical criteria) are, themselves, small organic molecules, having molecular weights typically below 300 Da, it is immediately evident why most of them could have been considered, in the recent past, unsuitable for MALDI analysis of LMW analytes. Considering, for instance, the archetype matrix 4-hydroxy-α-cyanocinnamic acid (CHCA), a plethora of matrix related ions are generated during laser irradiation. Apart from the matrix (M) protonated adduct $[M+H]^+$ at *m/z* 190.0499 and main fragment ions at *m/z* 172.0393 ($[M-H_2O+H]^+$) and *m/z* 146.0600 ($[M-CO_2+H]^+$), sodiated and potassiated adducts are also observed together with several matrix clusters ions covering the range from *m/z* 379.0925 ($[2M+H]^+$) up to *m/z* 1287.0864 ($[5M+4K-3H]^+$) [6]. It is also worth of note that the shift of the laser wavelength from 337 nm (N_2 laser) to 355 nm, provided by the recently introduced Nd:YAG solid-state lasers, results in even more intense CHCA matrix clusters. Although specific pre-treatments of the applied samples (e.g. washing of previously crystallized sample spots with ammonium-containing buffers) could significantly reduce the spectral abundance of matrix clusters, the matrix related background ions and the low accuracy/resolution of the old generation MALDI instruments, no doubt have discouraged the use of conventional matrices for LMWC analysis. Apart from significant instrumental improvements, several efforts have been made and several approaches have been proposed to partially suppress or even avoid matrix-related peaks such as, for instance, the use of matrix additives [7–9], and the synthesis of novel organic and inorganic matrices [10–14]. A separate mention is deserved by matrix-less

approaches such as desorption ionization on silicon (DIOS), pioneered by Siuzdak's group, which opened new horizons for small molecules analysis by laser desorption/ionization (LDI) MS [15].

Different deposition procedures of conventional matrices have been also exploited to boost MALDI MSI of LMW peptides, lipids, drugs and metabolites in tissues, cells and microorganisms. Recent advances in deposition strategies together with development of high spatial-resolution, high sensitivity MS instruments and novel matrices for MALDI MSI have been reviewed elsewhere [16–20]. Some recent "fundamental books" focusing on basic principles, instrumentation, methods and applications of MALDI MS, the interested reader should refer to, remain an invaluable source of information [21–25]. The mechanisms of ion formation in MALDI MS, still a matter of debate, have been the object of several dedicated reviews [26–32] that need continuously update as new insights in this topic are gathered [33–37].

The present review aims to provide a critical overview of the most recent achievements in the specific field of MALDI MS analysis of LMW analytes. The first section deals with the applications of classical MALDI matrices to LMWC through strategies such as the use of matrix additives, specific sample pre-treatments and matrix/analyte derivatization. New matrix deposition strategies, even if specifically devised for MALDI MSI experiments, are also shortly described since target analytes are typically LMW primary and secondary metabolites. The second (main) section deals with second generation organic matrices and is structured into sub-sections each focusing on a class/function of emerging matrices for LMW analytes, i.e., reactive matrices, matrices for negative analysis, binary, inorganic and hybrid organic/inorganic matrices, ionic liquids and electron transfer matrices.

2. First generation matrices for LMWC analysis

Representative examples of first generation matrices for UV MALDI (see **Figure 1**) are benzoic acid derivatives (*e.g.* DHB or 2,5 dihydroxybenzoic acid), cinnamic acid derivatives (*e.g.* CHCA), ferulic acid, sinapinic acid (SA) and 2,4,6-trihydroxyacetophenone (THAP). Interestingly, some of these matrices, originally developed for the analysis of proteins, peptides and oligonucleotides, are "*lucky survivors*" even for LMWC (despite the presence of potentially interfering matrix-related ions) mostly because of the introduction of last generation MALDI instruments. Indeed, as first demonstrated by Persike *et al.* [38], matrix signals can be conveniently used for the internal calibration, thus significantly improving mass accuracy (e.g. <5 ppm for acetylcholine). As a result of accurate mass measurement and high resolution offered by modern time-of-flight (TOF) instruments, the CHCA fragment ion $[M+H-CO_2]^+$ at *m/z* 146.061 could be resolved from the protonated acetylcholine (ACh) ion ($[C_7NH_{16}O_2]^+$) at *m/z* 146.117. ACh and Ch were successfully determined in mouse brain microdialysis samples. Persike and Karas [39] further confirmed that the sensitivity, resolution and mass accuracy of a last generation MALDI-TOF MS system permit to overcome the limitations posed by the matrix (CHCA) background demonstrating the simultaneous quantification of ten phenothiazines in spiked plasma samples. An atmospheric pressure MALDI high-resolution mass spectrometry (quadrupole/orbital trap hybrid analyzer) method has been described [40] for the simultaneous detection/quantitation of 26 triazines and triazoles in grapes using CHCA as matrix. MS and MS/MS spectra were acquired at a resolution of 35,000 FWHM (full width at half-maximum) and mass accuracy was typically within 5 ppm. The protonated adduct of atrazine-desethyldesisopropyl and cyprazine could be detected at *m/z* 146.0228 and 228.1011, respectively, in spite of the presence of matrix ions $[M+H-CO_2]^+$ and $[M+K]^+$ with

monoisotopic mass 146.0600 and 228.0058, respectively.

Among first generation matrices, DHB seems to be the most appropriate for lipid analysis [41] since, contrary to cinnamic and sinapinic acid, it gives a low yield of photochemically generated matrix ions. Porcari *et al.* [42] reported the characterization of lipid extracts of Atlantic sturgeon caviars using a DHB matrix in combination with a high resolution mass spectrometer. Yet, unless some special precautions are taken, for many other LMWC, these conventional matrices do not work as successfully as in the previous examples owing to matrix effects, signal interference, or suppression of the analyte signal.

2.1. Matrix additives/dopants, supported matrices, and new deposition strategies

One of the simplest way to minimize or even remove the matrix background spectral interferences is to exploit the well-known but often overlooked matrix suppression effect (MSE) as suggested by McCombie & Knochenmuss [43]. Since laser intensity and the matrix/analyte molar ratio are the most easily governable factors strongly influencing MSE, laser power and analyte concentration were systematically varied for many LMW (from 150 to 550) test analytes using two conventional matrices (DHB and CHCA) and finally evaluating a properly defined MSE score. Since high values of analyte-to-matrix molar ratios are required for MSE being effective, an inconvenient that can be encountered (when more than one analyte is present) is the simultaneous occurrence of analyte suppression effects (ASE).

Matrix background could be suppressed, more easily, in positive ion mode by adding CHCA with a quaternary ammonium (QA) surfactant [44, 45] such as cetyltrimethylammonium bromide (CTAB) as first demonstrated by Guo *et al.* [46] who also introduced the acronym “matrix suppressed laser desorption/ionization” (MSLDI). Since effective MSE can be achieved only above certain analyte/matrix molar ratios, it is suggested that in MSLDI, CTAB

could behave itself as an analyte during the LDI process. Within the two-step model of MALDI ionization, this implies a depletion of primary matrix ions by neutral analytes, via secondary ion-molecule reactions. In MSLDI a slight improvement in resolution was also achieved, probably due to cool ions (i.e. small plume) generation; however, MSLDI has a poorer detection limit which should make it most useful for screening of abundant LMWC.

One problem encountered in using QA salts as additive, is that not only the matrix ions but also the analyte signals could be unacceptably suppressed if the ratio of matrix/QA salt/analyte is not carefully optimized; an issue that is further complicated by the possibility that the optimal ratio might be dependent on the nature of matrix, analyte and QA salt. Mechanistic aspects of matrix and analyte suppression effects induced by QA salts, have been investigated [47]. It was suggested that the observed MSE and ASE could be rationalized by a cluster ionization model assuming that analytes and matrix ions, coexisting in the cluster, compete for the limited number of net charges available. In the presence of a sufficiently amount of QA ions, the net charges, will be removed thus resulting in the MSE and ASE. According to the authors 'the cluster ionization model' was not conflicting with the ionization *via* secondary gas-phase reactions and, ultimately, the final ions observed are the combined results of both mechanisms. Later on, Lou et al [48], using different model compounds and two different sample deposition, i.e. standard dried-droplets and modified thin layer methods, demonstrated that generation of gas-phase ions from charged matrix/analyte clusters is the ionization step likely responsible for matrix/analyte ion suppression effects. Ion suppression effects in multi-analyte mixture can be significantly reduced by caesium chloride addition to a DHB matrix as demonstrated by Popkova and Schiller [49] for lipid analysis in crude adipose tissue extracts where, in absence of added salts, phosphatidylcholines and phosphatidylethanolamines are suppressed by the most

abundant triacylglycerols (TAG). Comparison with other alkali chlorides provided evidence that the size of the alkali ion plays a role in ion formation yield and analyte fragmentation as well.

A different strategy to reduce/eliminate matrix interference, relies on the incorporation of the matrix (e.g., DHB) into a sol-gel polymeric structure as reported by Lin and Chen [50] or on matrices supported onto different organic/inorganic materials. An example is provided by 2',4',6'-trihydroxyacetophenone monohydrate (THAP) matrix supported on cyclodextrin that was successfully applied for the analysis of testosterone and diazepam [51]. In the presence of cyclodextrin, matrix fragment ions and alkali adduct ions were significantly suppressed in favour of $[THAP+H]^+$ thus reducing the background and amplifying the analyte signals. The same matrix was supported on lithium-substituted mordenite [52] and used for acetylsalicylic acid and phenobarbital analysis. The binding energies of Li^+ and H^+ to THAP, estimated by quantum-chemical models around 236 and 927 kJ/mol, respectively, account for Li^+ detachment from $[THAP+Li]^+$ producing $[analyte+Li]^+$ ions. The tested molecules were then preferentially ionized as lithium adducts avoiding the concurrent formation of protonated or other alkali metal adducts that would adversely affect sensitivity.

A similar mechanism can be invoked when neutral NaDHB and NH_4DHB salts were used as matrices for lipids analysis [53]. The cationization efficiency was improved avoiding the separation of lipid species into several ionization states. This allowed for simplified lipid spectra interpretation and detection of sterols and steryl esters not affordable by standard acidic DHB.

Lithium 2,5-dihydroxybenzoate (LiDHB) [54], lithium α-cyano-4-hydroxycinnamate, lithium sinapate and lithium salts of other organic aromatic acids [55] were synthesized and tested as potential matrices for MALDI MS of hydrocarbons, di- and tri-acylglycerols and wax esters.

Lithium vanillate offered the most remarkable increase in sensitivity (compared to LiDHB) for long-chain hydrocarbons and wax esters; moreover, the formation of homogeneous spots makes this matrix a potential candidate for MALDI MSI applications.

Novel deposition methods of conventional matrices have been essentially developed for MALDI MSI where thin tissue slices (prepared by cryo-section) are mounted on sample plates and fixed; then matrix is applied on the entire tissue or on specific regions of interest [56]. Gemperline *et al.* [57] compared three application techniques (namely airbrush, automatic sprayer and sublimation) of DHB and CHCA matrices for the metabolome analysis of *Medicago truncatula* root and nodule tissues. A comparison of mass spectra, provides a straightforward evidence that the peak patterns of matrix are greatly influenced by the application technique; airbrush deposition generated the most intense matrix related background that could significantly hinders metabolites detection. Moreover, when sample was analysed, the sublimation and automatic sprayer methods produced less pronounced analyte delocalization and an increased number of metabolites detected especially when using DHB.

Recently, an electric field-assisted scanning-spraying matrix coating system [58] was described. The generation of very small crystals (< 10 μm) of common matrices resulted in an enhancement of detection sensitivity and number of LMWC (fatty acids, nucleosides monophosphate, nucleosides and N-acetylneuraminic acid) could be detected in cancerous tissues. The same method was employed using other matrices (*i.e.,* quercetin for positive and negative ion detection and 9-aminoacridine (9AA) for negative ion detection) for the analysis of endogenous compounds in the cancerous and non-cancerous regions of human prostate cancer tissues; 152 metabolites, out of the 1091 overall detected and localized, exhibited differential distributions between the two tissue regions [59].

Small and homogeneous spots can be generated by electrowetting-assisted drying [60] on special functionalized e-MALDI target plates. This deposition method proved successful for the analysis of LMW drugs such as paracetamol, quinine, penicillin V, aspirin, ibuprofen, and fenofibrate. As clear from scanning electron microscopy (SEM) images (**Figure 2**) e-MALDI drop drying leads to very uniform crystallization of DHB which resulted in improved spot quality, enhanced sensitivity, reduction of the analytes' lateral diffusion in MSI experiments. Very small and regular crystals were generated by electrospray deposition which allowed for an intimate incorporation of analyte throughout the matrix layer. This method was applied for the analysis of peptides [61] and for MALDI FTICR imaging of lipids in mouse brain and liver cancer tissue section [62]. Interestingly, the electrospray deposition method was suitable for either various organic matrices or metallic nanoparticles (NPs), which made it unique for MALDI MSI analysis. A recently proposed sample deposition protocol exploits the inkjet technology to automatically drop cells and matrix solutions onto ITO glass plates whose surfaces were made hydrophobic by treating them with octadecyltrimethoxysilane. Membrane phospholipids (*e.g.* phosphatidylcholines) of printed cells were detected by MALDI MS using DHB as a matrix [63]. Similarly, a subcellular detailed investigation was achieved by a dual-positioner nanomanipulator workstation which permitted a concomitant extraction of organelle content and the co-deposition of analyte and matrix solution for MALDI analysis [64].

Since high quality MALDI MS images are heavily dependent on the matrix deposition step an open-source software and hardware device has been recently developed [65, 66] capable of delivering uniform and reproducible coatings even by unexperienced operators.

Finally solvent-free one-step automatic matrix deposition permitting homogeneous coverage of mouse brain tissue with 2-4 µm sized crystals of CHCA has been described by

Trimpin et al [67]. The advantages of these deposition strategies, over those employing additives, stems from their adaptability to various kind of matrices and the absence of further interfering signals due to their pure physical character. However, they are specifically designed for MALDI MSI experiments and often require a dedicated instrumentation.

2.2. High molecular weight matrices, matrix/analyte derivatization

One feasible alternative approach to elude the problem of matrix background in the low *m/z* range is to search for high molecular weight (HMW) matrices (see **Figure 3**). An example is given by 2,3,4,5-tetrakis(3´,4´-dihydroxylphenyl)thiophene (DHPT), which allows detection of a wide range of LMW amines (i.e., amino acids, peptides, vitamin B, β-agonists, alkaloid and aromatic amines) at femtomole level (Chen *et al.* [68]). Compared with CHCA, DHPT gives a mass spectrum almost devoid of peaks in low mass range, the sole matrix ions detected being the radical cation at *m/z* 516.087 (positive ion mode) and the deprotonated molecule at *m/z* 515.081 (negative ion mode). Also worth of mention in this framework, is a group of synthetic porphyrins having a high enough molecular weight to ensure absence of spectral interference for small molecules. A further advantage is that their laser absorption and proton affinity can be fine-tuned by changing the side chains on the central structure. Ayorinde *et al.* [69] first tested meso-tetrakis(pentafluorophenyl)porphyrin (F_{20}TPP) as a candidate MALDI matrix for the analysis of alkylphenol ethoxylates; F_{20}TPP was also successfully employed for several other applications such as the analysis of the fatty acid composition of vegetable oils [70], detection of sugars, ascorbic acid, citric acid and sodium-benzoate in beverages [71] and HIV protease inhibitor drugs in cell extracts [72]. The goal of shifting the matrix mass towards higher values could also be achieved by synthesizing suitable derivatives of a conventional matrix. Porta *et al.* [73], for instance, produced the

CHCA derivatives (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid (NpCCA) and (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid (AnCCA) pursuing a twofold purpose: (i) minimize the matrix spectral interference in the low *m/z* range ~~also~~ and (ii) obtaining more favourable proton affinity values. Indeed, AnCCA has no matrix ions below *m/z* 230 while NpCCA has only a few ions below this *m/z* value; an improvement *per se* only marginal compared to CHCA that, however, is compensated for by significantly improved signal-to-noise ratio (compared to CHCA) for 30% of the LMWC tested.

A feasible alternative to HMW matrices is to shift the analytes mass towards higher *m/z* values using appropriate derivatizing agents; by such an approach charged groups can be eventually introduced thus increasing the analyte's ionization efficiency. A representative example is given by *N*-phosphorylation reaction of natural amino acids and small peptides as proposed by Gao *et al.* [74]. Under mild conditions, the *N*-terminal and ε-amino group of lysine can be fully labelled with a neutral phosphoryl group originating a reduced number of side products and permitting the analysis of these target molecules in serum sample as *N*-phosphoryl derivatives. Compounds containing primary or secondary amine groups were easily derivatized by tris(2,4,6-trimethoxyphenyl)phosphonium acetic acid and *N*-hydroxysuccinimide esters. This derivatization reaction, allowing a limit of detection in the low femtomole range, was applied to quantify a mixture of antibiotics by investigating isotopically coded light and heavy derivatives [75]. Girard's reagents T and P have been employed for the derivatization of oxosteroids [76], steroids [77] and small oligosaccharides [78]. Hailat *et al.* [79] converted some selected sterols into their corresponding picolinyl esters, *N*-methylpyridyl ethers and sulphated esters before their analysis in mussels' extracts by MALDI MS using DHB and THAP conventional matrices. A nonreductive amination

reaction using aminopyrazine as co-matrix of DHB and derivatizing agent has been proposed for the characterization of oligosaccharides released from selected N-glycoproteins [80].

One drawback common to the above described labelling methods is that additional tedious purification steps are required implying that one of the strengths of MALDI (*i.e.* reduced sample manipulation) is lost. To prevent this problem, Rohmer e*t al.* [81] suggested to perform the derivatization reaction directly on the target plate using 3-aminoquinoline (3-AQ) as both labelling reagent and MALDI matrix. However, derivatization rates were found critically dependent on the amount of organic solvents required to guarantee high yields in the spot. To avoid this issue, a modified protocol was proposed introducing an ionic liquid matrix; mixing 3-AQ and CHCA allowed for high-sensitivity detection of glycans detached from oligosaccharides [82]. A distinctive approach, *de facto* involving analyte derivatization, is represented by reactive MALDI matrices that will be specifically treated later (*vide infra*).

3. Second generation matrices for small molecules analysis

An attractive strategy in the development of novel MALDI matrices consists in their rational design, whereby the core structure of classical matrices is purposely modified to achieve specific physo-chemical properties [14]. This represents the step forward towards the replacement of the mostly empirical approach to the matrix choice, typical of the early times of MALDI, with a rationally based one. The pioneers in the field of rationally designed (RD) matrices were Jaskolla and co-workers who synthesized numerous CHCA and DHB derivatives aiming to tune their proton affinity and improve analyte ionization efficiency. It was demonstrated that the PA could be reduced by incorporating electron withdrawing moieties, e.g. halogens, into the CHCA core structure [83]. Among the screened matrices, 4-chlorocyanocinnamic acid (ClCCA), obtained by replacing the hydroxyl group of CHCA with a

chlorine atom, was effective in increasing the sensitivity of tryptic digest of standard proteins. Indeed, because of their reduced PA, the halogenated matrices exhibit an improved protonation of weakly basic, neutral and acidic peptides. However, the addition of multiple electron withdrawing moieties [84] was not satisfactory due to a blueshift of the absorption wavelength and to ~~a~~ morphological changes (needle-like crystals were formed). A possible method to address these issues could be the mixture of two halogen-substituted compounds with similar PA or the addition of CHCA; in this case one matrix can be excited by the laser and can guarantee the proton transfer to the more halogenated matrix and finally to the analytes. Here, it is worth of mention that CClCA matrix has found interesting applications in the identification of lipids [85] and vitamin B [86][87]. The previously mentioned [73] NpCCA and (AnCCA) give a further example of RD matrices aimed to tune the PA of CHCA with a concomitant shift towards higher *m/z* values. These novel matrices showed a better S/N ratio and crystallization behaviour compared to CHCA. 4-phenyl-α-cyanocinnamic acid amide matrix was recently described for negative ion mode MALDI MSI of various lipid classes displaying a superior sensitivity and reproducibility compared to e.g. 9AA [88]. Fukuyama et al. developed alkylated DHB for analysis of hydrophobic peptides [89] that was later applied for the MALDI MSI of phospholipids in brain tissue [90].

Very recently, a library of 59 structurally related cinnamic acid derivatives was synthesized and potential MALDI matrices were assessed by analysing sulfatides, a class of anionic lipids abundant in brain [91]. The cinnamic acids currently used are *E*-cinnamic isomers; Salum *et al.* [92] reported the use of *Z*-sinapinic acid (SA) for MALDI MS of peptides. Compared with *E*-SA and CHCA, a smaller number of clusters was observed in the low *m/z* region allowing the rapid and sensitive detection of very short hydrophilic and hydrophobic peptides.

3.1. Reactive matrices

The so called “reactive matrices” can work simultaneously as MALDI matrices and as a derivatization agent to improve ionization yield, gain structural information, or enable analysis of small molecules by lowering their volatility or increasing their final *m/z* value. Reactive MALDI was first described by Zhang and Gross [93]; a mixture of anthranilic acid (AA), nicotinic acid and diammonium citrate (2:1:0.003) formed a Schiff base, namely an “in situ” MALDI matrix reacting with the basic sites of modified oligodeoxynucleotides. A mixture composed of 2,4-dinitrophenylhydrazine (DNPH), a well-known derivatizing agent, and DHB was reported by Brombacher *et al.* [94] for the analysis of seven corticosteroids separated by capillary liquid chromatography coupled (off-line) to MALDI MS. Such a mixture was reported as a “reactive matrix” which can be somewhat misleading since DHB played the usual role of a conventional MALDI matrix whereas reactivity (i.e., formation of steroid-dinitrophenylhydrazone derivatives) was merely ensured by the added DNPH [95]. Similarly DNPH and 4-dimethylamino-6-(4-methoxy-1-naphthyl)-1,3,5-triazine-2-hydrazine (DMNTH) have been successfully used as reactive matrices for the analysis of carbonyl containing compounds [96]. Note, however, that significant improvements in sensitivity and limits of detection can be only achieved using prolonged (48 h) reaction times and the assistance of a conventional CHCA matrix. Conversely, Schiller’s group has demonstrated that DNPH can be used as a genuine reactive matrix (i.e. no assistance by a conventional matrix) for the MALDI MS analysis of aldehydes after conversion into their corresponding hydrazones [97]. By such an approach, lipid oxidation products could be easily detected; at the same time, it was demonstrated that DNPH also enabled the detection of non-oxidized phospholipids. Other hydrazide and hydrazine reagents [98][99] were applied for the detection at parts per million (ppm) levels of small hazardous gases possessing aldehyde (formaldehyde, acetaldehyde,

propionaldehyde) or ketone (acetone, methyl ethyl ketone, and methyl isobutyl ketone) moieties.

The pyrylium cation is a six-membered heterocyclic ion consisting of five carbon atoms and one positively charged oxygen atom. A family of pyrylium salts was tested as derivatizing agents for the analysis of small molecules containing primary amine groups [100]. Among these, 2,4-diphenyl-pyranylium (DPP) was used as an effective reactive matrix to map the distribution of dopamine and amphetamine and to quantify a neurotoxin in brain tissue sections [100]. The aromatic compounds 2,4-dihydroxybenzaldehyde and 2,5-dihydroxyacetophenone were employed for the study of polyamines [101] while 1,2-phenylenediamine was selected for the analysis of ketocarboxylic acids as pyruvate [102]. Oligosaccharides were derivatized with a 2-hydrazinopyrimidine reactive matrix that can facilitate their ionization due to the presence of an electron-withdrawing *N*-heterocycle. This approach was applied to the analysis of neutral and sialylated oligosaccharides released from glycoproteins of biological samples. Moreover, their improved fragmentation allowed a detailed structural study by MS/MS [103].

A modified version of this strategy, named label-assisted LDI, employed a pyrene based boronic acid for the detection of various cis-1,2-diols. The boronic functional group could act as a molecular recognition probe towards *vic*-diols allowing their selective detection via LDI-MS in a single experiment. The strategy was applied for catecholamine neurotransmitters dopamine and epinephrine detection [104].

An example of RD reactive matrix, aimed to confer molecular recognition properties to the archetype matrix CHCA, was recently described [105]. 4-formyl-phenylboronic acid (FPhBA) was condensed with cyanoacetic acid by a readily accessible standard Knoevenagel condensation, resulting in the formation of [(E)-4-(2-cyano-2-carboxyvinyl) phenyl]boronic

acid (CCPBA); the α-cyanocinnamic acid core was preserved while the hydroxyl moiety was replaced by a boronic acid residue able to act as a molecular recognition probe. Although the UV-vis absorption spectrum of CCPBA showed a slight bathochromic shift compared to CHCA, a similar molar absorption coefficient value at the laser wavelength (337 nm) was observed. It was demonstrated that this matrix selectively recognizes vic-diols, α-hydroxyacids and amino alcohol compounds. For instance, small diols such as glycerol (MW = 92), 1,2-ethandiol (MW = 62) or 1,3-propanediol (MW = 76), that are metabolic markers of chronic pathologies or contaminants of food commodities, could be readily detected by MALDI MS at level as low as 50 pmol/spot. Moreover, the proposed matrix first allowed the selective detection of fluoride anions even in the presence of equimolar amounts of other halides. To show the effectiveness of the CCPBA matrix, a human urine sample was simply diluted (1:10 v/v) with water and directly analysed obtaining a highly informative spectrum where several metabolites could be tentatively assigned. It is worth of note that, under the same conditions, a conventional matrix as 9-AA gave a spectrum containing only matrix-related peaks.

An unpredicted matrix reactivity towards some analytes has also been described [106][107]. For instance, thiosalicylic acid (TSA), known for its reducing properties, behaves as a reactive matrix towards disulfide bond producing TSA-adducted peptides. Comparison with MALDI mass spectra obtained using conventional matrices permits counting of disulfide bonds in the investigated peptides [106]. An unusual case of “unwanted reactive MALDI” has been described by Chendo et al. [107] using standard acidic matrices (e.g. 2,5-DHB, DCTB and dithranol among others) to assist ionization of nitroxide-terminated poly(4-vinylpyridine). Either covalent or non-covalent adducts with some of the investigated matrices were formed

depending on the MALDI source pressure; a mechanism was also proposed to explain the matrix/polymer covalent adduct formation.

3.2. Matrices for negative ion mode

9-Aminoacridine (9AA) was introduced for the analysis of LMWC (as phenols, carboxylic acids, sulfonates and alcohols) [108] in negative ion mode where proton transfer is typically dictated by differences of the gas-phase basicities of deprotonated analyte and deprotonated matrix [109]. Then 9AA has found extensive use for the analysis of phytohormones [110], bile acids in urine [111] and plasma [112], oligosaccharides [113], and phospholipids [114][115]; worth of mention are also metabolomic studies [116] [117] and the analysis of various natural resins and varnish samples from cultural heritage objects [118]. In some cases, however, matrices other than 9AA provided even better results in negative ion mode MALDI. Norharmane, for instance, permitted a 100-fold improvement, compared to 9AA, in the limit of detection of the bacterial endotoxin lipid A and phospholipids in tissues infected with *Francisella novicida* [119].

Other new matrices purposely designed for negative ion MALDI MS analysis of small molecules have been described, such as 2-mercaptobenzothiazole [120], N-(1-naphthyl)ethylenediamine dinitrate [121], 1,5-diaminonaphthalene [122], 2,5-diaminonaphthalene [123], 4-phenyl-α-cyanocinnamic acid amide [88] and quercetin [124]. 1,5-Diaminonaphthalene (DAN), has been reported for the analysis of phospholipids (PL), in both negative and positive ion modes [125], and LMW metabolites from corn leaf sections [122]. As far as the PL is concerned, it seems that DAN provided significantly higher signals for phosphatidylethanolamines whereas 9AA seems to preferentially ionize phosphoinositol (PI) species. DAN hydrochloride salt was used in MALDI imaging of liver, brain, and kidneys tissues from mice permitting to visualize the distribution and change of small metabolites

including metal ions, amino acids, carboxylic acids, nucleotide derivatives and lipids [126]. DAN exhibits an interesting behaviour acting as a reduction agent, leading to the cleavage of disulphide bonds and thus facilitating in-source decay fragmentation of proteins [127]. DAN generates mainly c- and z-series ions by N–Cα bond cleavage while 3-hydroxy-4-nitrobenzoic acid, a recently proposed oxidizing matrix, generated numerous a- and d-series ions [128] giving a complete picture of peptide pattern fragmentation. It has recently demonstrated that DAN can also function as electron transfer matrix (see below).

Several other matrices for negative ion MALDI-MS analysis of small molecules have been described such as 1-naphthylhydrazine hydrochloride [129], N-(1-naphthyl)ethylenediamine dinitrate [121], 2,5-diaminonaphthalene [123], 2-(2-aminoethylamino)-5-nitropyridine [130] and nifedipine (2,6-dimethyl-3,5-dicarbomethoxy-4-(2-nitrophenyl)-1,4-dihydropyridine) [131] but their use is quite limited to very specific applications.

Ammonia-treated N-(1-naphthyl) ethylenediamine dihydrochloride has been employed as a matrix in negative ion mode to directly quantify free fatty acids (FFAs) in serum samples. By constructing multiple point internal standard (IS) calibration curves using a C17:0 FA as IS it was possible to demonstrate as FFAs in hyperglycemic patient sera were significantly higher than healthy controls [132]. A similar organic matrix, N-(1-naphthyl) ethylenediamine dihydrochloride, was helpful in quantifying sulfated oligosaccharides such as chondroitin-4-sulfate in biological samples using deuterium-labeled ISs [133].

A significant breakthrough in this field is, no doubt, represented by the introduction of the so called “proton sponges” as MALDI matrices. The Alder’s proton sponge [134] (see **Figure 4**), 1,8-bis (dimethylamino)naphthalene (DMAN) was rediscovered and first proposed by Svatos’ group in the more general framework of a rational protocol for matrix selection based on Brønsted–Lowry acid–base theory [135] [136]. A distinctive feature of a proton

sponge (B) is due to a different behaviour compared to classic MALDI matrices; being a very strong base (pK_{BH}^+ ≈ 18 in acetonitrile) the proton sponge is supposed capable of deprotonating an acidic analyte AH in the liquid-phase thus forming an ion pair $[A\text{-}H]^-$ /$[B+H]^+$. The acid–base driven equilibrium between the ion pair and the AH/B complex in the liquid-phase is mirrored in the crystal phase and is responsible for the amounts of observed $[A\text{-}H]^-$ ions in the gas-phase upon laser irradiation. To highlight this mechanism, the acronym "matrix-assisted ionisation/laser desorption" (MAILD) was then proposed [136]. As DMAN itself does not undergo deprotonation in the gas-phase, a further distinctive feature of such a matrix is that it is entirely ionless (in negative ion mode) thus representing a paradigm for MALDI MS analysis of small-molecules as deprotonated anions. Indeed, DMAN proved particularly effective for negative ion mode analysis of several LMW acidic metabolites (e.g., carboxylic acids, fatty acids, amino acids, vitamins, plant and animal hormones) involved in important metabolic pathways such as Krebs cycle, fatty acid and glucosinolate biosynthesis.

In the field of lipidomics, DMAN revealed effective for lipid fingerprinting of Gram-positive (*Lactobacillus sanfranciscensis* and *Lactobacillus plantarum*) microorganisms by intact cells MALDI MS [137]; free fatty acids, mono-, di- and triglycerides, phospholipids, glycolipids and cardiolipins (CLs), spanning a *m/z* range from 187 (deprotonated azelaic acid) to 1496 ([CL 75:5 $-H]^-$) were readily detected. The most noticeable drawback of DMAN is represented by its high sublimation rate under high-vacuum conditions which make it poorly suitable for long lasting experiments such as MALDI MSI. Other proton sponges, stronger than DMAN, such as 1,8-bis(tetramethylguanidino)naphthalene (pK_{BH}^+ ≈ 25 in acetonitrile) were proposed for the determination of perfluorooctanesulfonates and perfluorooctanoic acid in water samples [138]. Twelve azahelicenes, displaying proton affinities comparable to those of classical proton sponges, have been tested as MAILD/MALDI matrices for the analysis of

fatty acids and organic acids in a wide range of samples [139]. At least one of the azahelicenes investigated operates as a MALDI matrix (i.e., analyte deprotonation in the gas phase) whereas the most promising compound, i.e. 1,14-diaza[5]helicene, was found a MAILD matrix better than DMAN.

Novel rationally designed matrices, retaining the 1,8-substituted naphthalene ring of DMAN, could be synthetized aimed to outperform the closely-related "predecessors". A representative example is provided by 1,8-di(piperidinyl)naphthalene (DPN), a low vapour pressure and ionless matrix, differing from the parent DMAN for the presence of piperidinyl substituents that increase the distance between the di-nitrogen center-chelated proton and a deprotonated anion, and steric repulsion [140]. According to the MAILD mechanism an increased distance is assumed to facilitate the dissociation of the ion pair, thus increasing the analyte signal intensity. Since the basicity of DPN and DMAN are roughly the same, it is supposed that the higher ionization efficiency exhibited by DPN could result from kinetic effects. Indeed proton shielding in $[DPN+H]^+$ by the two piperidinyl groups should reduce the rate at which (in the gas-phase) the proton is exchanged back to the analyte anion resulting in an improved ionization. It is worth noting that DPN is capable of ionizing, with very low sensitivity, some compounds lacking an acidic proton such as glucose that can be detected as deprotonated molecule. Being vacuum stable, DPN can be proficiently used for imaging experiments.

A further example of vacuum stable proton sponge is provided by 3-(4,5-bis(dimethylamino)napthalen-1-yl)furan-2,5-dione (4-maleicanhydridoproton sponge or "MAPS" – **see figure 4**) [141] synthesised by reacting, at room temperature, DMAN with bromomaleic anhydride according to the Tyler´s reaction [142]. Despite structural modification due to maleic anhydride moiety introduction in the DMAN structure, the pK_{BH^+}

of MAPS (18.0 in acetonitrile) was comparable to that of DMAN (18.6); at the same time the ionless property of DMAN was retained. However, the higher vacuum stability of MAPS, compared to DMAN, makes it better for MALDI MSI experiments of small molecules. MAPS was thus successfully employed to obtain ionic maps of lactate, 2-hydroxyglutarate and chloride anions (*m/z* 89, 147, 35, respectively) in an aggressive brain tumour tissue (*glioblastoma multiforme*) as shown in **Figure 5**.

A noteworthy advance in the field of rationally designed matrices possessing outstanding basicity has been recently provided by our group [143]. Superbasic proton sponges having a 1,8-bisphosphazenylnaphthalene (PN) core unit and diverse P-amino and -alkyl substituents such as dimethylamino, pyrrolidino, tris-(pyrrolidino)phosphazenylbis(pyrrolidino), methyl, n-butyl, isopropyl, and cyclo-pentyl have been recently synthesized and characterised [144]. Such proton sponges display exceedingly high pK_{BH+} values ranging approximately from 30 to 32, in acetonitrile, depending on the substituents. Such outstanding basicity makes PNs potential candidates as negative ion MAILD/MALDI matrices offering a promising tool for producing deprotonated molecules from otherwise hardly ionizable small compounds. Indeed, the possibility of producing deprotonated species, with virtually absent fragmentation, from compounds lacking acidic protons such as steroids, sterols, fatty alcohols and saccharides was recently demonstrated. For instance, using a TPPN [1,8-bis(trispyrrolidinophosphazenyl)] matrix (see **Figure 5),** cholesterol could be detected as intact deprotonated molecule at *m/z* 385.348 and quantified at levels as low as 3 pmol/spot or 6 nM. Other representative small molecules such as stearyl alcohol, β-estradiol, 17-α-ethinylestradiol, nomegestrol, ergocalciferol, cholecalciferol, β-sitosterol, campesterol and sucrose could be deprotonated as well. Cholesterol, fatty acids, lysophospholipids and phospholipids could also be analysed in complex samples such as egg yolk and brain tissue

extracts. Contrary to DMAN, which is completely ionless in negative ion mode, the tested superbasic proton sponges produce few, clearly recognizable, matrix-related fragment ions indicating that they are not entirely photostable under laser irradiation. Moreover, TPPN was experimentally proved to be, contrary to DMAN, a vacuum-stable matrix suitable for MSI experiments. Among superbasic proton sponges, TPPN proved also capable of deprotonating neutral carbohydrates providing an efficient and simple way to produce gas-phase anions [145]. Note that, negative ion mass spectra tend to be much more informative than positive ion counterpart, because of predominant cross-ring cleavage ions. To overcome the inability of conventional matrices to produce deprotonated molecules several approaches, often unsatisfactory for neutral saccharides, have been proposed such as the use of matrix additives (chloride salt, sulphuric acid [146], and benzenesulfonic/p-toluene sulfonic acids [147]) and chemical derivatization using 2-aminobenzoic acid [148] or 2-aminoacridone [149]. A further approach is represented using nonconventional matrices (β-carboline alkaloids) such as nor-harman working well for neutral cyclomaltooligosaccharides [150] but not for linear oligosaccharides [151] or harmine doped with ammonium chloride [152]. In this framework, the use of TPPN opens the way for structural characterization of neutral mono-, di-, tri- and tetra-saccharides, cyclodextrins, and saccharide alditols by MALDI-MS/MS.

3.3. Binary, hybrid (organic-inorganic) and nanomaterial-based matrices

Binary matrices were first proposed by Solouki [153]; 4-nitroaniline/coumarin and fructose/DHB matrix mixtures were investigated aimed at improving ion yield in MALDI Fourier -Transform Ion Cyclotron Resonance (FTICR) MS for the analysis of biomolecules such as bovine insulin and lysozyme (use of melittin as internal calibrant also improved mass accuracy). A binary matrix specifically intended for MALDI MS analysis of LMWC was

described by Guo and He [154]; indeed a CHCA/9AA mixture produced fewer background interferences compared to single matrix in both positive and negative ion mode. After a screening of many LMWC, it was concluded that only analytes with pKa values outside the range of pKa of the binary components could be proficiently detected. As a result, other binary matrices were developed for the analysis of small biomolecules. Shanta *et al.* [155] tested a combination of 3-hydroxycoumarin and 6-aza-2-thiothymine for the analysis of drugs or a combination of DHB, CHCA with a mixture of trifluoroacetic acid and piperidine for the identification of phospholipids in rat brain tissue sections [156]. The binary matrix composed of 2,5-DHB and 2,6-DHB was shown to improve the detection of glycans released from ovalbumin by PNGase F: 16 glycans could be detected compared to 10 and 9 glycans detected using 2,5-DHB and 2,6-DHB single matrix, respectively [157]. A binary matrices composed of DMAN and 9-aminoacridine (9-AA) was used for the direct analysis of whole bacterial cells in Gram positive and Gram negative microorganisms [158]. About fifty major membrane components including free fatty acids, acylglycerols, phospholipids, and glycolipids were easily detected in negative ion mode. Ionizing properties of N-butyl-4-hydroxy-1,8-naphthalimide (BHN) were evaluated using model compounds such as O-acetyl-L-carnitine hydrochloride, oxytocin and small peptides. BHN was found to improve co-crystallization of target analytes when mixed with DHB; compared to CHCA the BHN/DHB binary matrix provided a lower background and improved signal-to-noise ratio in the determination of small molecules desorbed from rat brain tissue slices by positive ion MALDI MS [159]. Carbon dots (CDs)-9AA binary matrix was used for nucleosides, amino acids, oligosaccharides, peptides, and drugs analysis in human urine exhibiting excellent reproducibility compared with 9AA matrix; it was argued that CDs likely act as a matrix additive capable of suppressing 9AA ionization [160].

Hybrid organic-inorganic matrices consisting of e.g. organic matrix and metal nanoparticles have been proposed with the aim to attain a synergic effect and outperform other MS methods for small molecule analysis. Lin *et al.* [161] first proposed this type of strategy combining conventional matrices (DHB and CHCA) with polymeric metal nanoparticles (MNP) for the detection of small drugs as salicylamide, mefenamic acid, ketoprofen, and prednisolone. The hybrid matrix provided high ionization efficiency with low background noise for the analysis of small target molecules in a complex mixture. An hybrid composed of immobilized silica and DHB on iron oxide magnetic nanoparticles for MALDI MS analysis of diverse LMW organic and inorganic compounds has been reported [162]. A hybrid material obtained by covalent attachment of an ionic liquid, composed by CHCA and 3-aminopropyltriethoxysilane (APTES), to TiO_2 nanoparticles has been reported as a novel MALDI matrix for LMWC such as amino acids and dopamine. The rationale beyond such an approach is an expected synergic effect exhibited by the ionic liquid (improved ionization efficiency) and the nanomaterial (desorption/ionization process facilitated by the large surface area). Indeed, compared to CHCA, the hybrid matrix provided reduced background and improved sensitivity [163]. A mixture of DHB and Fe_3O_4 nanoparticles was successfully used to alleviate the issue of triacylglycerol (TG) ion suppression by phosphatidylcholine (PC) [164] in MALDI MSI of maize seed cross-sections. A hybrid organic-inorganic materials composed of CHCA covalently attached to amorphous silica (prepared by standard sol-gel chemistry) was characterized as a matrix for LDI analysis (as protonated and cationized adducts) of diverse synthetic peptide mixtures covering a 550-1300 Da mass range [165]. An hybrid matrix composed of CHCA covalently bound (trough APTES) to mesoporous silica was synthesized and used to analyze twelve quinolone antibiotics and three pesticides [166].

Inorganic (e.g. silicon- and metal-based) nanomaterials are largely studied in the framework of LMWC analysis by surface assisted laser desorption ionization (SALDI) techniques (and variant) that are outside the scope of this review. Silicon-based matrices include silicon nanospot arrays [167, 168], silicon nanowires [169] and silicon nanopillars [170], while a huge number of metal matrices is reported based on gold [171–175] and silver substrates [176, 177] or metal oxides as titanium oxide [178–180], zinc oxide [181] and lithium oxide [182]. For further information, the reader should refer to recent dedicated reviews [10, 12, 176, 183]. A recent overview of desorption/ionization mechanisms in SALDI MS is also available [184].

A particular mention deserves graphene as matrix for the analysis of LMWC (amino acids, polyamines, nucleosides, anticancer drugs, and steroids) by MALDI MS [185]. The graphene matrix functions as analytes trapping substrate and energy receptacle for laser radiation, providing highly efficient desorption/ionization free of matrix related ions and without significant fragmentation. Graphene as MALDI matrix was also employed for nitropolycyclic aromatic hydrocarbons determination in $PM_{2.5}$ samples using 9-nitroanthracene-d_9 as the internal standard [186]. To overcome limitations to spatial resolution imposed by traditional matrix applications in MALDI MSI, a two-dimensional sheet of graphene was deposited directly on top of soybean leaves and rat brain tissue samples via a “dry transfer” process that ensures absence of analyte diffusion. Several classes of LMWC (small peptides, lipids, glycosylated metabolites) are desorbed and ionized, with minimal background interference from the matrix. [187].

Graphene oxide (GO) has been employed as matrix for negative ion mode MALDI MSI of mouse brain tissue sections [188]; 190 lipids and 22 LMW metabolites were overall detected whereas in situ MS/MS provided structural identification of 69 lipids. Compared with 9AA

and N-(1-naphthyl) ethylenediamine dihydrochloride matrices, GO revealed especially effective for fatty acids and sphingolipids. Metabolite heterogeneity in viable and necrotic tumour regions of mouse breast cancer tissue could be also highlighted. Interestingly, the signal-to-noise (S/N) ratios of low-abundance phospholipid classes that are frequently suppressed by the presence of most abundant and easy to ionize phosphatidylcholines in can be selectively improved, in positive mode MALDI MSI, using GO matrix [189]. Functionalized GO was used as a matrix for the detection of dopamine in cerebrospinal fluid [190] and for lipids analysis by TOF-SIMS [191]; GO modified with 4-vinylphenylboronic acid was used to selectively capture compounds with vicinal diols [192].

Finally, a short mention is deserved by hybrid nanomaterials such as metal–organic frame-works (MOFs) namely coordination bonded networks consisting of metal ions as connectors and organic ligands as linkers [193]. An interesting aspect of MOF is that they can be designed (through the appropriate choice of the organic ligand) to act both as matrices for LDI MS of small molecules and adsorption material for simultaneous analyte enrichment as demonstrated for phosphopeptides [194] and environmental pollutants such as Benzo(a)pyrene and perfluorinated compounds [195].

3.4. Ionic liquids

Ionic liquid matrices (ILMs) for MALDI MS stem from general research on ionic liquids defined as non-molecular salts having a melting point below 100 °C or at or below room temperature in the case of the so-called room temperature ILs (RTILs). ILs possess negligible vapor pressure and good electrical conductivity; they are thermally stable, non-explosive, non-flammable and are capable to dissolve a wide range of analytes. Armstrong et al. [196] first investigated the potential use of a number of ILs as MALDI matrices. ILMs have the potential to overcome many of the limitations of solid matrices; in particular they do not

require co-crystallization with the analyte and produce homogeneous solutions thus avoiding search for “hot spots” and greatly improving quantitative analysis [197]. A second generation of ILMs was introduced by Crank and Armstrong [198] who examine over 100 cation/anion pairs aimed at designing ILs possessing different properties. The most common examples of ILMs consist of equimolar mixtures of conventional MALDI matrix compounds (e.g. DHB, CHCA, SA) together with organic bases (e.g., pyridine, tributylamine, aniline just to name few). ILMs have been successfully applied to a variety of molecules including peptides [199][200], proteins [201], phosphopeptides [202], oligosaccharides [203][204], phospholipids [205]. Some review papers [206] [9] and a recent book chapter [207] are available.

Here representative applications of ILMs in the framework of MALDI MS detection, imaging and quantitation of LMWC will be highlighted. Indeed, an outstanding property of several ILMs is their suitability for LMWC since, compared to the classical solid matrix, background signals are significantly suppressed by the charged organic base. For instance, CHCA/Aniline ILMs revealed a highly versatile matrix for amino acids, peptides, proteins, lipids, phospholipids, synthetic polymers, and sugars [208]. ILMs have been employed as sample probe and matrix for liquid-liquid microextraction and ionization of phospholipids in soybean [209] or vegetable oils [210]. MALDI MS analysis of phosphopeptides in the low femtomole range was achieved using a solidified ionic liquid matrix consisting of 3-AQ and CHCA [211]. The ILMs formed by CHCA/triethylammonium (or diisopropylammonium) were used to analyse, with minimum sample preparation, 14 pharmaceutical drugs in different tablets, capsules and solutions providing a simple procedure for detecting drug counterfeits [212].

Some examples of applications of ILMs for LMWC imaging are worth of mention. CHCA based ionic liquids were employed for the neuronal single cell profiling of lipid species by

matrix enhanced-secondary ion mass spectrometry (ME-SIMS) [213] whereas CHCA/butylamine and DHB/butylamine ILMs were successfully used for MALDI MSI of lipids in mouse liver and cerebellum tissue sections [214].

Three ionic liquid matrices based on 2,5-DHB (i.e., 2,5-DHB/Aniline, 2,5-DHB/Pyridine, and 2,5-DHB/ 3-acetylpyridine) were tested for MALDI MSI of lipids in human ovarian cancer biopsies [215]. RTILMs consisting of equimolar mixture of mefenamic acid and different bases (aniline, dimethyl aniline, pyridine, and 2-methyl picoline) were tested for their ability to ionize different and wide classes of compounds such as drugs, carbohydrate, and amino acids and found useful for MALDI MS analysis of lysates of pathogenic bacteria [216]. Recently, MALDI MS quantitative analysis of fructo-oligosaccharide mixtures in rice noodles, using two ILMs consisting of equimolar mixture of DHB with N-methylaniline or N-ethylaniline has been reported [217].

A detailed investigation of 27 ILMs for analyte quantification by MALDI MS was reported by Moon et al. [218] who found that some specific requirements should be met in order to ensure spot-to-spot spectral reproducibility.

3.5. Electron transfer matrices

Common matrices can putatively form radicals, but they scarcely exchange electrons with analyte, being the competing proton transfer the favoured process. The electron transfer (ET) mechanism can become important when compounds with low ionization potentials are examined. The choice of matrices with ionization energies (IE) higher than analyte could promote electron transfer secondary reactions, thus avoiding the occurrence of proton transfers that usually induce the formation of matrix clusters. Accordingly, ET matrices could be potentially useful for LMWC analysis. McCarley *et al* [219] found that anthracene and terthiophene could work as ET matrices for metallocenes as ferrocene. A two-photon

ionization of matrix was proposed, followed by ET from the analyte to the matrix radical cation. This process is possible only if the ionization potential (IP) of the analyte is lower than the matrix one (in this example $IP_{anthracene}$ = 7.44 eV vs $IP_{ferrocene}$ = 6.71 eV). Anthracene, pyrene, and acenaphthene were utilized as nonpolar matrices for the analysis of polybutadiene, polyisoprene, and polystyrene samples of various average molecular weights [220], 9,10-diphenylanthracene was used as ET secondary reaction matrix for the analysis of retinol and chlorophyll [221] and tetrathiafulvalene was used for pigments detection [222].

Non protic 2-[(2 E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene] malononitrile (DCTB) has proven to be an excellent ET matrix for labile compounds such as substituted fullerenes, due to a very low onset of ion production [223]. Such a non-polar and aprotic matrix was also used in MALDI MS analysis of coordination compounds, organometallics, porphyrins, phthalocyanines, carbohydrates, calixarenes, and macrocycles. A comparison of DCTB performance with that of dithranol, 2,5-DHB, and 2,4,6-THAP matrices was provided [224].

Eight phenylenevinylene oligomers (PVs) were synthesized and characterized as potential ET matrices for UV-MALDI. Indeed, molar absorptivities (at 355 nm) are similar or higher than those of CHCA and DCTB, calculated IP values range from 6.88 to 7.96 eV and positive ion mode LDI MS shows the presence of PV radical cation free of clusters, adducts, or protonated species. Four selected PVs were tested as ET MALDI matrices for porphyrins, phthalocyanines and polyaromatic compounds achieving, in the most favourable case, LOD in the low fmol range [225].

Effective ET matrices are typically aprotic compounds; however it has been recently demonstrated that even matrices with basic residues as DAN can work as ET matrices for the analysis of chlorophylls [226] and bacteriochlorophylls [227]. Indeed, DAN drastically

outperformed other ET matrices since it avoided demetallation and phytol–ester linkage fragmentation usually observed in the previous MALDI MS analysis of chlorophylls.

Conclusions and outlooks

Recent achievements in the design and applications of conventional and novel matrices, specifically addressing the challenging issue of MALDI MS analysis of LMWC, are presented and discussed. The outlook for the future is promising. Definite evidences are provided that MALDI MS represents a powerful and invaluable analytical tool also for small molecules analysis, including their quantification, thus opening new exciting applications in most disparate fields including food and environmental chemistry, biomedicine, metabolomics and imaging mass spectrometry to name just a few. The large degree of molecular heterogeneity of LMWC (paradigmatic is the field of lipidomics) precludes the search for a universal matrix; on the other hand, demands on quantitative MALDI MS and MALDI MSI applications ask for matrices possessing, in addition to the classic requisites, precise physicochemical features such as homogeneous crystallization, small crystal sizes, high vacuum stability.

First generation archetype matrices (e.g. CHCA and DHB) have been adapted to LMWC analysis using additive/dopants or matrix/analyte derivatization but they tend to be replaced by second generation (rationally designed) matrices. Rationally designed matrices possessing molecular recognition properties have been proposed that are expected to find interesting new applications for targeted MALDI MS analysis.

Ionic liquid matrices, whose properties can be tailored for specific applications by varying the nature of the acidic and basic counterparts, can improve spot homogeneity, spot-to-spot reproducibility and analyte quantitation. Furthermore, many liquid matrices can be operated

in both positive and negative ion modes, thus helping profiling complex mixtures such those encountered in metabolomics/lipidomics studies. RTILs are promising matrices also for MALDI MSI since spatial resolution is not limited by the size of the matrix crystals and their enhanced durability allows for a high number of repetitive shots on one tissue position.

Matrices for negative ion mode have significantly contributed to extend MALDI MS to LMWC analysis and research efforts in this specific topic are far from being considered exhaustive. Prototypical is the discovery of completely ionless proton sponges' matrices and superbasic proton sponges capable of deprotonating compounds lacking acidic protons such as steroids, sterols, fatty alcohols and saccharides. Moreover, vacuum stability of such matrices makes them potential candidates for MALDI MSI experiments. Proton sponges provides an exemplary case of compounds originally intended as catalyst for organic synthesis that have found surprising applications as MALDI matrices. It is not the sole example; indeed, many inorganic, organic and hybrids nanomaterials, originally designed and developed for applications completely outside the field of MS, are more and more considered for their applications as MALDI matrices for LMWC analysis and imaging. Carbon based nanomaterials (i.e. graphene, two-dimensional graphene, graphene oxide) appear particularly promising even for imaging experiments. Phenylenevinylene oligomers, provide the most recent example of a class of materials, originally intended for applications in the field of organic electronics and whose properties can be rationally designed, that has been discovered as ET matrices for LMWC. The continuous development of new advanced organic/inorganic materials presumably will continue to drive the attention of mass spectrometrists towards their possible applications in MALDI MS to address continuously challenging analytical problems. It is not hazardous to foresee that the search for new MALDI matrices will likely be an endless story.

Acknowledgment

This work was supported by the project PONa3 00395/1 “BIOSCIENZE & SALUTE (B&H)” of the Ministero Italiano per l’Istruzione, l’Università e la Ricerca (MIUR).

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Table 1. List of the described organic matrices with their preferred ionization mode, their applications and relevant references.

Matrix	Preferred Ionization mode	Application	Ref.
CHCA	Positive	Acetylcholine, phenothiazines	38,39
	"	Triazines	40
	"	Phenolic acids, caffeine	43
	"	Trialanine, caffeine	44
	"	Pharmaceutics	66
CHCA/9-aminoacridine	Pos/negative	LMW compounds	155
CHCA/TFA/piperidine	"	Phospholipids in brain	157
CHCA/3-APTES on TiO_2	Positive	Amino acids, dopamine	164
DHB	Positive	Amino acids	45
	"	Lipids in caviar	42
	"	Drugs	60
	"	Peptides	61
	"	Lipids in brain and liver	62
	"	Cell lipids, organelles	63,64
	Positive (Li^+)	Nonpolar lipids, hydrocarbons	54
	Positive (Na^+)	Lipid	53
	Positive (Cs^+)	Adipose tissue	49
CHCA/Aniline	Positive	Amino acids and lipid in food	209
CHCA/butylamine	Positive	Phospholipids in food	210
CHCA/tributylamine			211
Triethylammonium or diisopropylammonium and CHCA	Positive	Pharmacological drugs	213
1-methylimidazole/CHCA	Positive	Lipid species in single cell profiling	214
Tripropylamine/CHCA			
DHB/fructose	Positive	Bovine insulin	154
DHB/TFA/piperidine	Pos/Negative	Phospholipids in brain	157
2,5-DHB/2,6-DHB	Positive	Glycans	158
Alkylated DHB	Positive	Hydrophobic peptides	89
		Lipids in brain	90
DHB/2,4-dinitrophenylhydrazine (DNPH)	Positive	Corticosteroids	94
DHB and CHCA with polymeric metal nanoparticles	Positive	Salicylamide, mefenamic acid, ketoprofen	162
Silica/DHB on iron oxide magnetic	Positive	Solar cell devices, dendrimers, glycolipids	163
Silica/CHCA		Quinolone antibiotics	167
DHB/N-butyl-4-hydroxy-1,8-naphthalimide	Positive	O-acetyl-L-carnitine hydrochloride, oxytocin and endogenous metabolites	160
DHB on Fe_3O_4 nanoparticles	Positive	Triglycerides, PL in maize	165
DHB with N-methylaniline and N-ethylaniline	Positive	Fructooligosaccharides in rice	218
Glycerol/CHCA	Positive	Peptides	200
Glycerol/CClCA	"	Peptides	201
Butylamine/DHB	"	Proteins	202
Pyridine or Butylamine/DHB	"	Phosphopeptides	203
Me(imidazolium)/CHCA,	"	Sulfated oligosaccharides	204
$BuNH_4$/DHB	Pos/Negative	Oligosaccharides	205
1,1,3,3-tetramethylguanidium/CHCA	"	Phospholipids	206
1-methyl imidazolium/CHCA			
THAP	Positive (Li^+)	Testosterone, diazepam	51
	"	Acetylsalicylic acid, barbital	52
Lithium vanillate	Positive (Li^+)	Wax ester, hydrocarbons	55
Quercetin	Positive	Metabolites in prostate tissue	59
	Pos/Negative	Lipids in adrenal gland	124
2,3,4,5-tetrakis(3´,4´-	Positive	Amino acids, peptides, vitamin B,	68

dihydroxylphenyl)thiophene		β-agonists, alkaloid, aromatic amines	
Meso-tetrakis(pentafluorophenyl)porphyrin	Positive	Alkylphenol ethoxylates Fatty acid in oils Sugars, ascorbic acid, citric acid, sodium-benzoate in beverages HIV protease inhibitor drugs	69 70 71 72
(2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid (AnCCA)	Positive	Pharmaceuticals	73
3-aminoquinoline (3-AQ)	Pos/Negative	Glycans, oligosaccharides	81,82
4-chlorocyanocinnamic acid	Positive	Peptides Lipids Vitamin B12	83 85 86,87
4-phenyl-α-cyanocinnamic acid amide	Negative	Lipids	88
Cinnamic acid derivatives	Negative	Sulfatides in brain	91
Z-sinapinic	Positive	Small peptides	92
Anthranilic and nicotinic acid	Negative	Oligodeoxynucleotides	93
DNPH/4-dimethylamino-6-(4-methoxy-1-naphthyl)-1,3,5-triazine-2-hydrazine (DMNTH)	Positive	Carbonyl containing compounds	96
DNPH	Positive	Lipid oxidation products Gaseous aldehydes and ketones	97 98
2-hydrazinoquinoline	Positive	Gaseous aldehydes and ketones	98, 99
2,4-diphenyl-pyranylium	Positive	Dopamine and amphetamine in brain	100
2,4-dihydroxybenzaldehyde and 2,5-dihydroxyacetophenone	Positive	Polyamines	101
1,2-phenylenediamine	Positive	Ketocarboxylic acids	102
2-hydrazinopyrimidine	Pos/Negative	Neutral and sialylated oligosaccharides	103
(E)-4-(2-cyano-2-carboxyvinyl) phenyl]boronic acid	Negative	Vic-diols, α-hydroxyacids, fluoride	105
Thiosalicylic acid	Positive	Disulfide bonds in peptides	106
9-aminoacridine	Negative	Metabolites in prostate tissue Phenols, sulfonates and alcohols Phytohormones Bile acids in urine and plasma Oligosaccharides Phospholipids Metabolomic Natural resins and varnish	59 108 110 111,112 113 114,115 116,117 118
9AA/ DMAN	Negative	Membrane lipids	159
9AA/Carbon dots	Positive	Nucleosides, amino acids, oligosaccharides, peptides, and drugs	161
Norharmane	Negative	Endotoxin lipid A and phospholipids Oligosaccharides	119 151
2-mercaptobenzothiazole	Negative	Lipid in egg	120
N-(1-naphthyl)ethylenediamine dinitrate	Negative	Oligosaccharides, peptides, metabolites, explosives	121
2,5-diaminonaphthalene	Negative	Lipid in human colon	130
1,5-diaminonaphthalene	Negative	Metabolites from corn leaf Phospholipids Imaging of liver, brain, and kidneys Peptides	122 125 126 127
1,5-diaminonaphthalene	Neg/Pos	Chlorophyll, bacteriochlorophyll	229,230
3-hydroxy-4-nitrobenzoic acid	Negative	Peptides	128
1-naphthylhydrazine hydrochloride	Negative	Glucose and homogentisic acid	129
2-(2-aminoethylamino)-5-nitropyridine	Negative	Phospholipids	131
Nifedipine	Negative	Polyphenols	132
1,8-bis (dimethylamino)naphthalene	Negative	Carboxylic acids, fatty acids, amino acids,	136,137

		vitamins, plant and animal hormones Lipids in microorganisms	138
1,8-bis(tetramethylguanidino)naphthalene	Negative	Perfluorooctanesulfonates and perfluorooctanoic in water	139
1,14-diaza[5]helicene	Negative	Fatty acids and organic acids	140
1,8-di(piperidinyl)naphthalene	Negative	Imaging experiments	141
4-maleicanhydridoproton sponge (MAPS)	Negative	Lactate, 2-hydroxyglutarate and chloride in brain tissue	142
1,8-bis(trispyrrolidinophosphazenyl)PS	Negative	Cholesterol, fatty acids, lipid Neutral saccharides	144 146
Harmine	Negative	Saccharides	153
4-nitroaniline/coumarin	Positive	Bovine insulin	154
Anthracene, terthiophene	Pos/Neg	Metallocenes	220
Pyrene, acenaphthene	Pos/Neg	Polybutadiene, polyisoprene, polystyrene	221
9,10-diphenylanthracene	Pos/Neg	Retinol, chlorophyll	222
Tetrathiafulvalene	Pos/Neg	Pigments	223
Trans-2-[3-(4-t-butyl-phenyl)-2-methyl-2-propenylidene] malononitrile	Pos/Neg	Fullerenes, coordination compounds, organometallics	224, 225
Phenylenevinylene oligomers	Pos/Neg	Porphyrins, phthalocyanines and polyaromatic compounds	226

Captions to Figures

Figure 1. Representative structures of typical first-generation matrices. Legend. **SA**: Sinapinic acid; **CHCA**: α-Cyano-4-hydroxycinnamic acid; **DHB**: 2,5-Dihydroxybenzoic acid; **DTN**: Dithranol or 1,8,9-Anthracenetriol; **THAP**: 2',4',6'-Trihydroxyacetophenone; **3-HPA**: 3-Hydroxypicolinic acid.

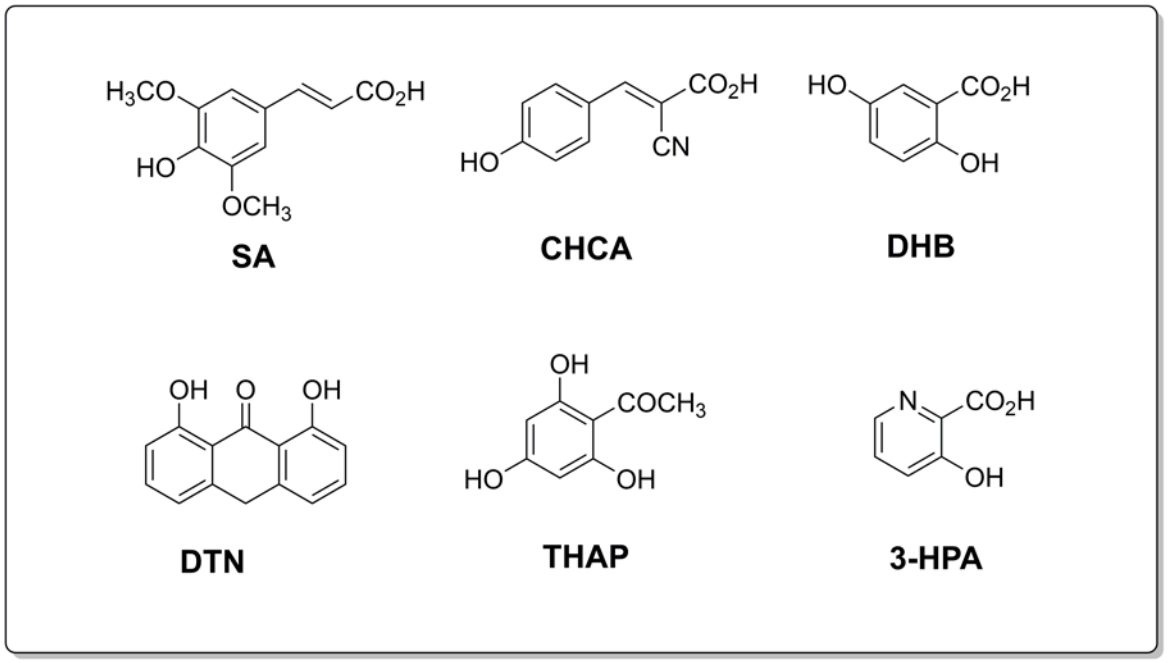
Figure 2. Top row: SEM images of dried samples of DHB matrix and paracetamol (10:1 molar ratio) with conventional (a) and with e-MALDI (b) drop drying (Thick scale bars: 0.5mm). Bottom row: same comparison for DHB matrix with quinine (10:1 molar ratio): c) conventional drop drying; d) e-MALDI (Thick scale bars: 0.25mm). Images were obtained side by side on the same target plate. Reproduced with permission from [60]. Copyright American Chemical Society, 2016.

Figure 3. Structures of some representative high molecular weight matrices. Legend. **DHPT**: 2,3,4,5-tetrakis(3´,4´-dihydroxylphenyl)thiophene; **AnCCA**: (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid; **NpCCA:** (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid; **F_{20}TPP**: meso-tetrakis(pentafluorophenyl)porphyrin.

Figure 4. Structures of some archetypal proton sponge matrices. Legend. **DMAN**: 1,8-bis (dimethylamino)naphthalene; **TMGN**: 1,8-Bis(tetramethylguanidino)naphthalene; **TPPN**: 1,8-bis(tripyrrolidinylphosphazenyl)naphthalene; **DPN**: 1,8-di(piperidinyl)naphthalene; **MAPS**: 4-maleicanhydridoproton sponge.

Figure 5. Structures of representative electron transfer matrices. Legend. DAN: 1,5-Diaminonaphthalene; **9,10-DP-ANT**: 9,10-Diphenylanthracene; **ANT**: Anthracene; **TTF**: Tetrathiafulvalene; **TER**: Terthiophene; **DCTB**: Trans-2-[3-(4-t-butyl-phenyl)-2-methyl-2-propenylidene] malononitrile.

Figure 6. MALDI Imaging results of a human diffuse glioma tissue section using the novel matrix MAPS. a) Optical (left) and H&E stained (right) sections were graded by a pathologist. Subsequent MALDI-Imaging showed the spatial distribution and regions of interesting spectra corresponding to chloride ions (b), lactate (c) and 2-hydroxyglutarate (d) in the tissue sections. Reproduced from Ref.141 with permission from The Royal Society of Chemistry.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CO2H
HO
OCH3
SA
CO2H
HO
CN
CHCA
HO
CO2H
OH
DHB
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O
OH
DTN
OH
COCH3
HO
OH
THAP
N
CO2H
OH
3-HPA

Figure 1

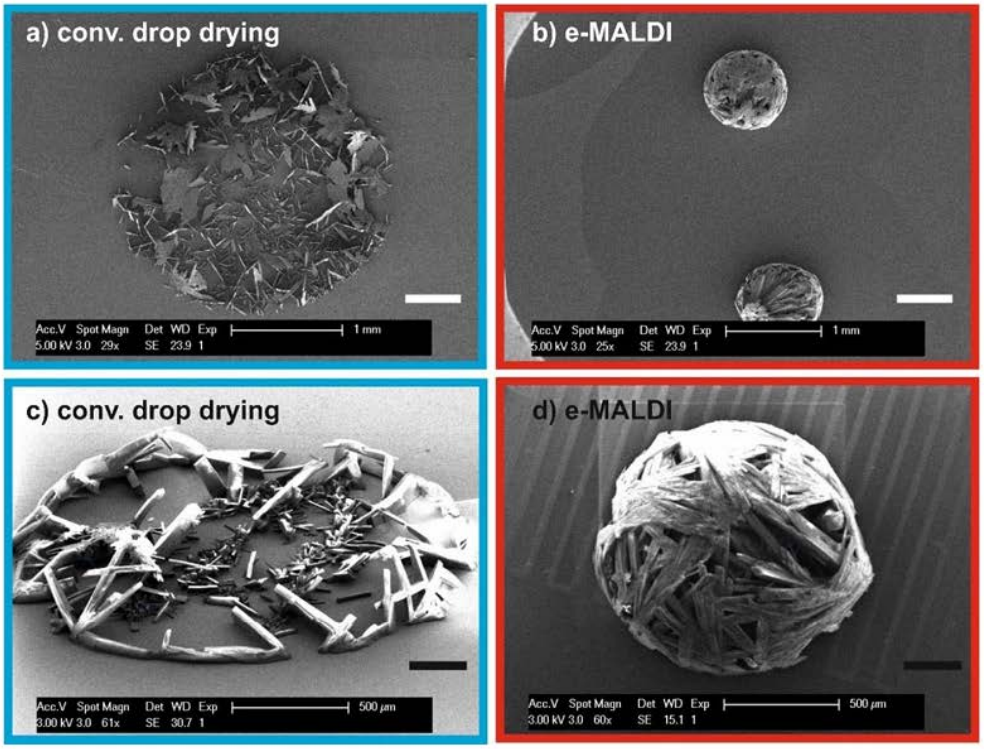
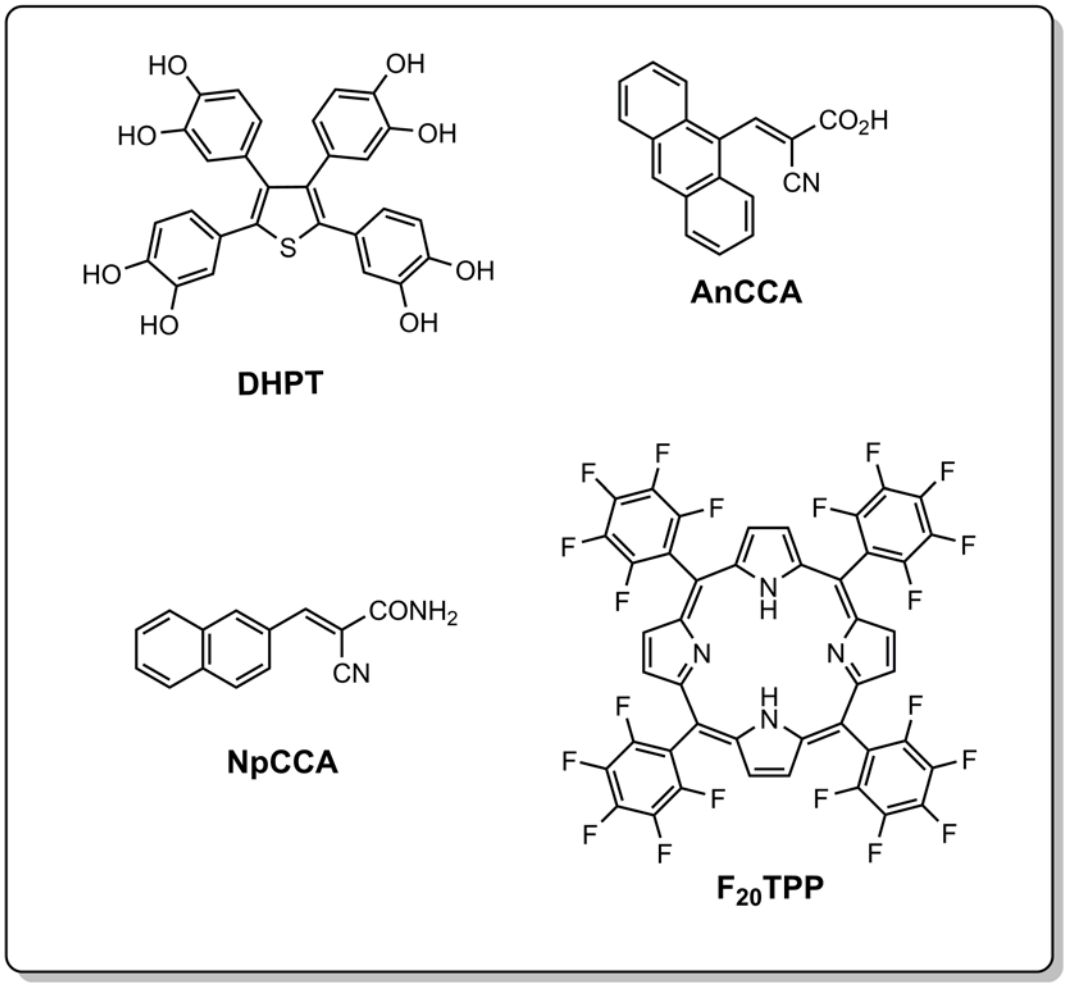

a) conv. drop drying
Acc.V Spot Magn Det WD Exp 1 mm
5.00 kV 3.0 29x SE 23.9 1
b) e-MALDI
Acc.V Spot Magn Det WD Exp 1 mm
5.00 kV 3.0 25x SE 23.9 1
c) conv. drop drying
Acc.V Spot Magn Det WD Exp 500 µm
3.00 kV 3.0 61x SE 30.7 1
d) e-MALDI
Acc.V Spot Magn Det WD Exp 500 µm
3.00 kV 3.0 60x SE 15.1 1

Figure 2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HO
OH
OH
S
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HO
OH
OH
DHPT
CO2H
CN
AnCCA
CONH2
CN
NpCCA
F20TPP

Figure 3

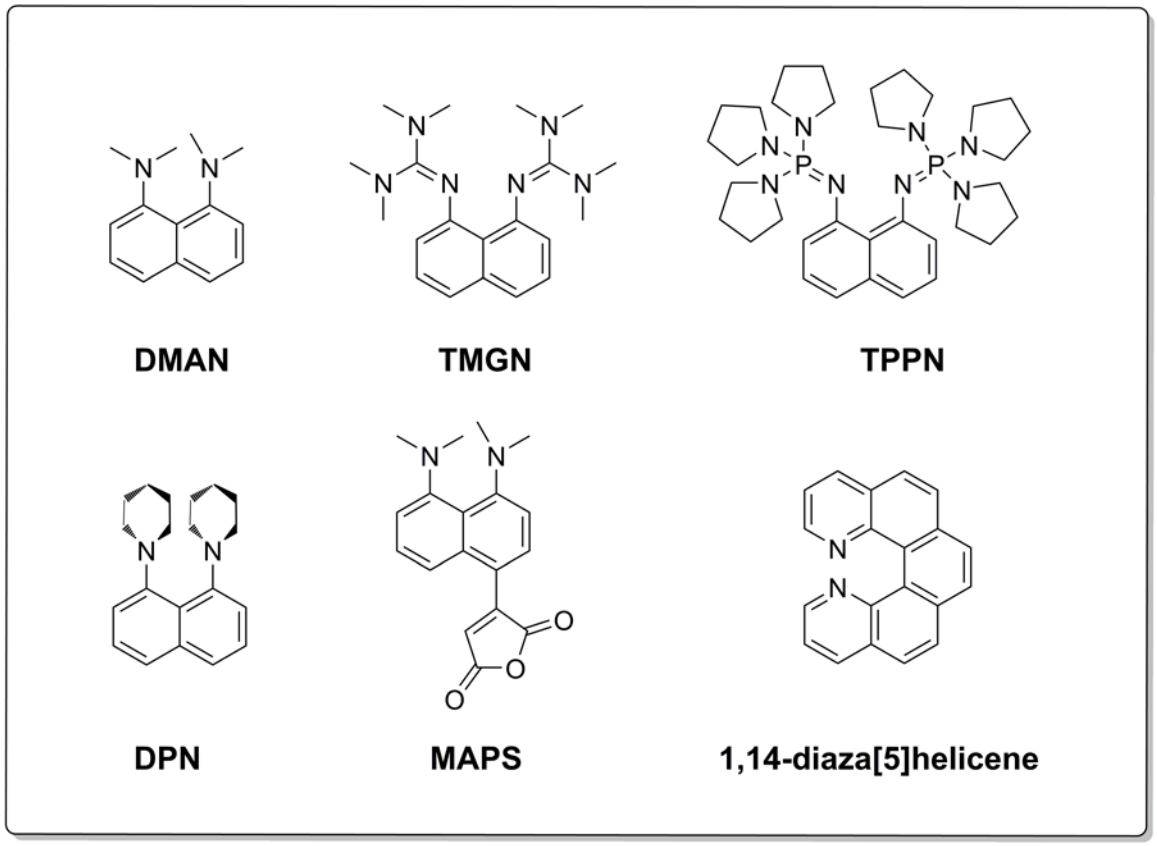

Figure 4

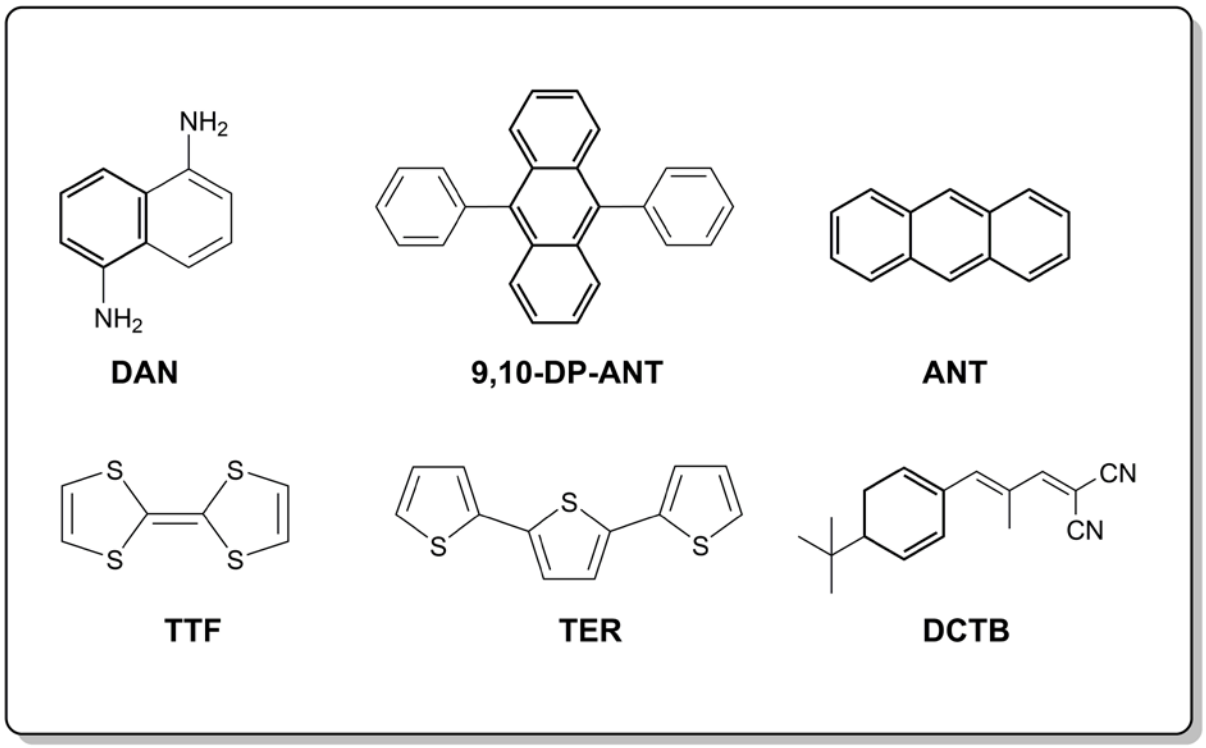

Figure 5

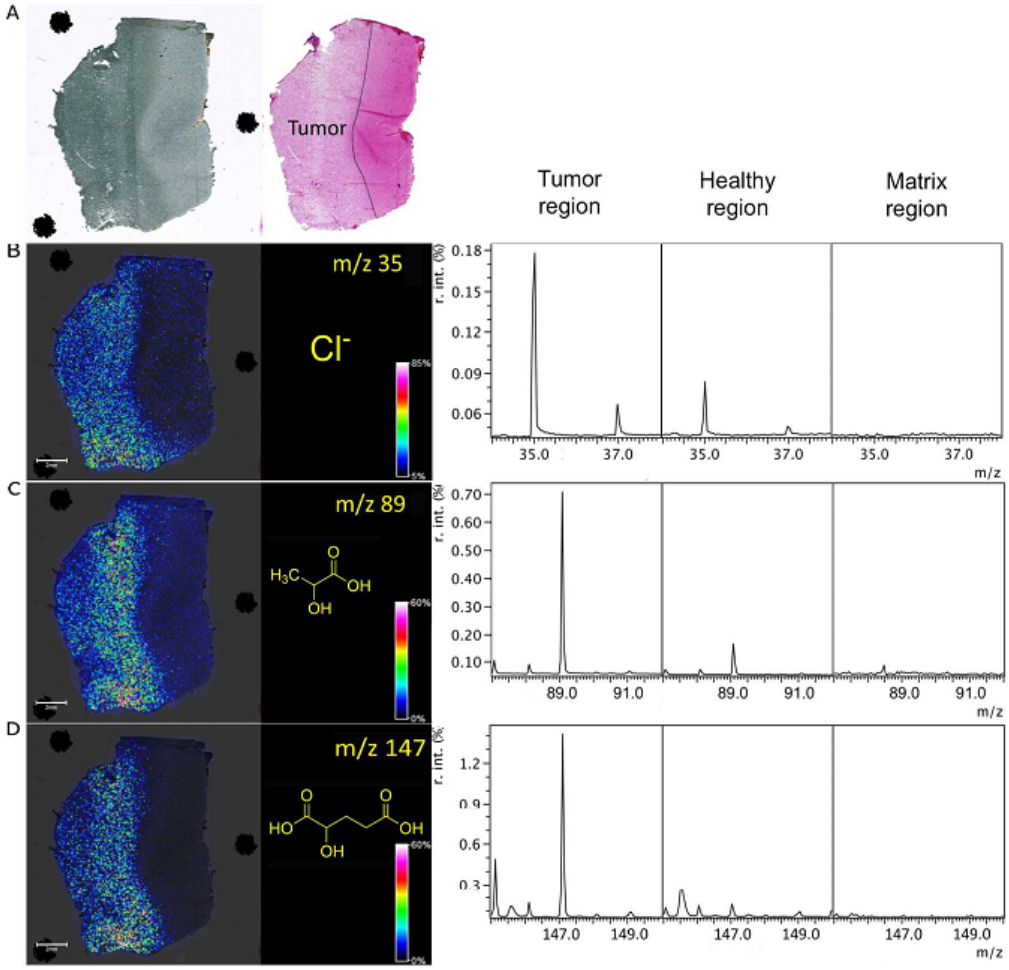
A
Tumor
Tumor region
Healthy region
Matrix region
B
m/z 35
Cl-
r. int. (%)
m/z
C
m/z 89
H3C
OH
OH
D
m/z 147
HO
OH
OH

Figure 6