

Chapter 2

MALDI Profiling and Applications in Medicine

Ed Dudley

Abstract Matrix-assisted laser desorption ionisation (MALDI) mass spectrometry allows for the rapid profiling of different biomolecular species from both biofluids and tissues. Whilst originally focussed upon the analysis of intact proteins and peptides, MALDI mass spectrometry has found further applications in lipidomic analysis, genotyping, micro-organism identification, biomarker discovery and metabolomics. The combining of multiple profiles data from differing locations across a sample, furthermore, allows for spatial distribution of biomolecules to be established utilising imaging MALDI analysis. This chapter discusses the MALDI process, its usual applications in the field of protein identification via peptide mass fingerprinting before focusing upon advances in the application of the profiling potential of MALDI mass spectrometry and its various applications in biomedicine.

2.1 MALDI Mass Spectrometry

Matrix-assisted laser desorption ionisation (MALDI) mass spectrometry (MS) represents an ionisation process first described in the mid-1980s and was developed by two independent research groups to the point at which MALDI mass spectrometers became commercially available in the early 1990s. The MALDI process involves the mixture of the analyte in solution with a “matrix” solution and co-crystallisation of both matrix and analyte together. During ionisation a laser (usually with a UV-nanometre wavelength) is fired at the mixture and causes desorption of the

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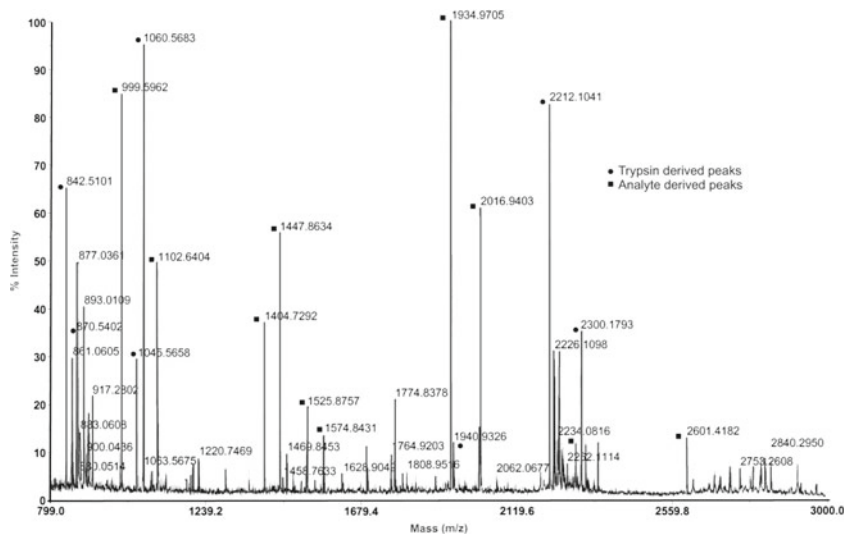
matrix and analyte into the gas phase. It is thought that the matrix is protonated first and thus protonates the analyte during the MALDI process. One major advantage of MALDI over other forms of ionisation, such as electrospray ionisation (ESI), is that usually singly charged ions are detected for the analyte even at increased mass/charge ratios. Comparatively ESI generates many multiply charged ion species for higher molecular weight protonated molecules which complicate the mass spectra generated and require deconvolution of the mass spectra in order to determine the molecular weight of the protonated molecule. MALDI ion sources are usually combined with time of flight (ToF) tubes for ion separation. The ToF tubes are utilised either in linear mode (in which ions pass once the full length of the ToF tube) or in reflectron mode (in which ions are reflected at the far end of the ToF tube and so pass through two times the length of ToF tube before reaching the detector). Reflectron mode analysis will provide a greater mass resolution but is limited to 5–10 kDa m/z ions whilst linear mode analysis can detect masses over 100 kDa and has a greater sensitivity. Considerations when undertaking a MALDI MS experiment are the choice of matrix to be mixed with the sample, how the sample and matrix solutions are mixed and the solvents used to solubilise both matrix and analyte. With reference to protein/peptide profiling and analysis, α -cyanohydroxycinnamic acid (CHCA) is the most commonly utilised matrix for peptide analysis by MALDI MS whilst proteins are thought to ionise more effectively using sinapinic acid (SA) as the matrix. Both are used in concentrations of around 10 mg/mL and therefore represent a considerable excess of matrix molecules to analyte molecules in the resulting co-crystallised mixture. As a result of this the lower end of the mass spectrum (less than 800 Da) is dominated by ions arising from the matrix itself and is therefore usually not studied during analysis. The solvent used for solubilising the matrix and mixing with analyte also differs between peptide and protein analysis with sinapinic acid dissolved in a solvent with a lesser acetonitrile content for protein analysis compared to CHCA for peptide analysis. The main purpose of this is to prevent proteins potentially precipitating due to the acetonitrile present which would prevent effective co-crystallisation of matrix and analyte. More recently, novel matrix materials have been investigated in order to determine whether improvements could be gained in ionisation efficiency. Much of the research has focussed on using alternative matrices in order to avoid low m/z ions which arise from the matrix itself and therefore extend the mass window of analysis for MALDI MS further into lower m/z regions. For this purpose graphene (a few layers of graphite) was utilised and allowed for the analysis of nonpolar polymethylmethacrylate—approximately 650 Da [12], whilst hydroxyflavones allowed for low molecular weight lipid profiling and also required less laser energy for efficient ionisation of the analytes [90]. Further advances in the analysis of peptides by MALDI MS have considered whether improvements in sensitivity can be accomplished by chemical derivatisation of the peptides prior to analysis. 1-(3-Aminopropyl)-3-butyliimidazolium bromide (BAPI) was utilised to derivatise the C terminal carboxyl groups of peptides prior to analysis and was reported to result in a 42-fold improvement in sensitivity and the derivatisation product was demonstrated to be stable for at least 1 week [78]. As an alternative, the phosphorylation of the N terminus of the peptides was also investigated as this

was selective for the N terminal lysine of tryptic peptides and due to the improved proton affinity provided by the additional phosphate group attached to the peptides an increase in sensitivity was again reported [25].

In relation to proteomic analysis, the most common application of MALDI MS is in undertaking peptide mass fingerprinting in order to identify an unknown protein and whilst this is not the topic of this chapter it is worth quickly reviewing. Peptide mass fingerprinting analyses the tryptic digest (usually) of a single protein and measures the m/z of the resulting tryptic peptides accurately (within at least 10 ppm). The resulting mass list of peptide m/z values can then be compared to a database of theoretical protein digest endproduct peptides and their singly charged m/z values and a protein identification suggested alongside a probability score for the identification—an example is given in Fig. 2.1. More recently, MALDI sources have been attached to ToF–ToF mass analysers which allow tandem mass spectrometry to be undertaken on peptides. This generates fragment ions (B and Y ions) alongside the mass of each peptide and therefore allows for more accurate identification of more complex protein mixtures; however, this area is not the focus of this chapter. In this chapter, we discuss the application of MALDI MS analysis in providing profiles of peptides and proteins from biological samples and how this type of profiling analysis has been more recently expanded to nucleic acid and metabolite analysis.

2.2 Sample Preparation for Protein/Peptides for Protein Profiling

Protein profiling has been applied to various biological fluids; however, the majority of studies have utilised plasma or serum as their starting material. Whilst serum and plasma are reasonably easy to collect in a clinical setting they do present the researcher with a large dynamic range of protein concentrations with up to 70 % of the protein present being represented by serum albumin and up to 25 % comprising globulin proteins [56]. These high abundance proteins are of little value for profiling purposes and can obscure the ability of analytical techniques to study low abundance proteins within the sample and therefore various sample preparation protocols have been developed and tested in order to be able to study lower abundance proteins by MALDI MS protein profiling. One obvious solution to the issue of high abundance proteins is to selectively remove these from the sample prior to analysis. A recent study compared immunodepletion columns that allowed for the removal of the 7 and 14 most abundant serum proteins prior to MALDI MS analysis and found that the removal of the 14 most abundant proteins and application of 2',4'-dihydroxyacetophenone as the matrix utilised for ionisation allowed for the most reliable protein profile to be produced [21]. One issue regarding such immunodepletion columns is their cost and the time consuming nature of the protocol. One column can usually be used to deplete up to 200 serum samples one at a time and each depletion requires a number of steps to prepare the column, deplete the sample,



Protein identification: Phosphoglycerate kinase

m/z submitted	m/z matched	Delta ppm	Sequence of peptide
999.5959	999.5991	3.1855	(K) FSLAPLVPR (L)
1102.6402	1102.637	2.6429	(K) RPFAAIVGGSK (V)
1220.7472	1220.747	0.6248	(K) VILSTHLGRPK (G)
1404.7299	1404.737	5.3725	(K) ELDYLVGAVSNPK (R)
1447.8636	1447.867	2.8167	(K) FLKPSVAGFLLQK (E)
1525.8751	1525.884	5.9406	(K) GVSLLLPTDVVVADK (F)
1573.8428	1573.843	0.6144	(K) GVTTHIGGGDSVAAVEK (V)
1933.9712	1933.977	3.0853	(K) LASLADLYVNDAFGTAHR (A)
2015.9488	2015.952	1.6474	(R) ADLNVPLDDNQITITDDTR (I)
2285.1848	2285.181	1.5930	(K)VGVAGVMISHISTGGGASLELLEGGK (V)
2600.4121	2600.403	3.3117	(K) AQGLSVGSSLVVEEDKLELATELLAK (A)

Fig. 2.1 An example of peptide mass fingerprinting spectra and the interpretation returned upon database searching

elute bound proteins and regenerate the column prior to the next sample being processed. A further complication may arise due to potential co-depletion of other proteins associated with albumin in serum samples [5]. The most common alternative approaches to serum preparation prior to MALDI MS analysis are to utilise

microscale tips with peptide/protein binding stationary phases incorporated or magnetic beads with a range of immobilised chemistries. The most commonly applied technologies are C18 reverse phase stationary phases for microscale tip purification which allows usually for the study of low molecular weight peptides and C8 reverse phase magnetic bead purification of low-to-medium molecular weight proteins. Whilst reverse phase stationary phases are the most commonly utilised for these types of sample preparation, other chemistries are available which exhibit differing affinities for different subsets of proteins. These include cation and anion exchange stationary phases, mixed ion exchange resins, immobilised lectin stationary phases (for purification of glycopeptides/proteins) and immobilised metal affinity technologies (for purification of phosphopeptides/proteins and anionic moieties). Example MALDI spectra after microscale tip and bead separation for peptide and protein profiling, respectively, are given in Fig. 2.2. In theory, the combination of all of these different preparation approaches would allow for the fractionation of serum samples and a more comprehensive survey of proteins present by MALDI MS analysis would result. These technologies have not only been applied to serum but also been optimised for the analysis of peptide/protein profiles from other biofluids. A recent study optimised the application of reverse phase microscale tip purification of peptides from serum and found that reproducible protein profiles detailing over 400 individual peaks could be determined [9]. Whilst the application of magnetic bead sample preparation to urinary peptide/protein profiling analysis noted that for ion exchange and immobilised metal purification the pH of the urine to 7 was required in order to allow reproducible recovery of analytes and whilst some inter-day variation was noted between samples, this variability was reduced by normalising the protein content of the urinary sample purified to set amount of protein (3.5 μg per sample) [22]. Similarly, magnetic bead purification protocols were applied to profiling of cerebrospinal fluid and it was determined that samples were stable for 6 h at room temperature and 3 days at 4 $^{\circ}\text{C}$ prior to purification and analysis; however, contamination with high albumin or globulin levels had a detrimental effect on the protocol and resulting mass spectra [8]. Of the two techniques (microscale tip and magnetic bead purification), the microscale tips have been reported as offering an improved and more reproducible protein exhibiting an average CV of 10 % of the recorded relative abundance for over 100 peptide/protein peaks in any given profile [84]. As an alternative approach, the fact that the majority of the high abundance proteins in serum are of comparatively high molecular weight has been used as a characteristic upon which to base a depletion protocol. Ultrafiltration of serum with filters with molecular weight cut-offs of 50 and 30 kDa has been applied as a methodology in order to allow for the study of low abundance proteins/peptides [3]. Such protocols commonly dilute the serum with acidic solutions (2 % trifluoroacetic acid for example) in order to dissociate peptides and proteins bound to albumin. Further processing of the sample after ultracentrifugation, such as desalting, has been reported to further improve protein coverage especially within the 3–20 kDa mass range [4, 26]. As an alternative approach, differential precipitation utilising ammonium sulphate and different organic solvents was tested for the preparation of calf serum for MALDI analysis [85]. The different fractions obtained presented different

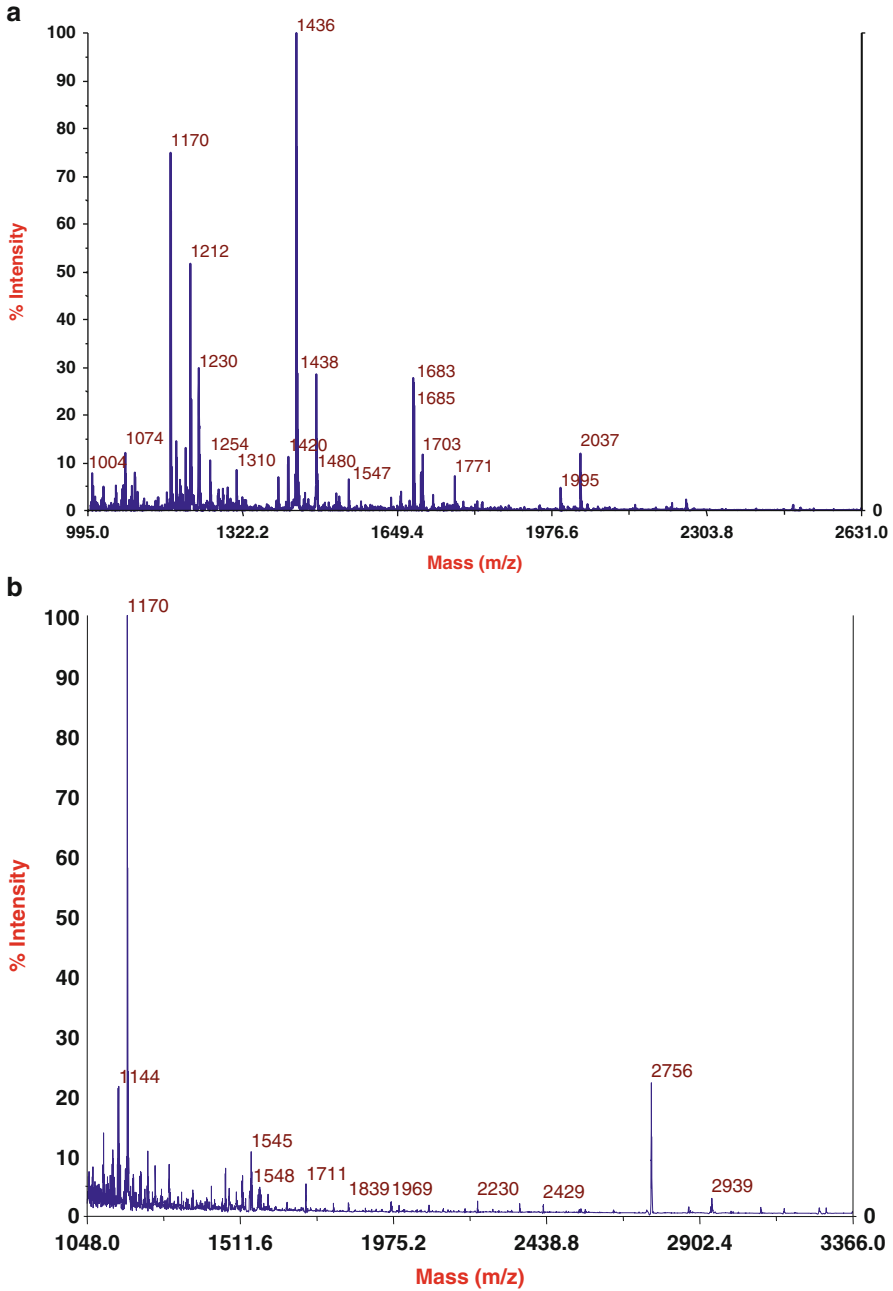


Fig. 2.2 MALDI protein and peptide profiling. (a) Global peptide profiling. (b) MALDI phosphopeptide profiling. (c) Global protein profiling. (d) MALDI phosphoprotein profiling

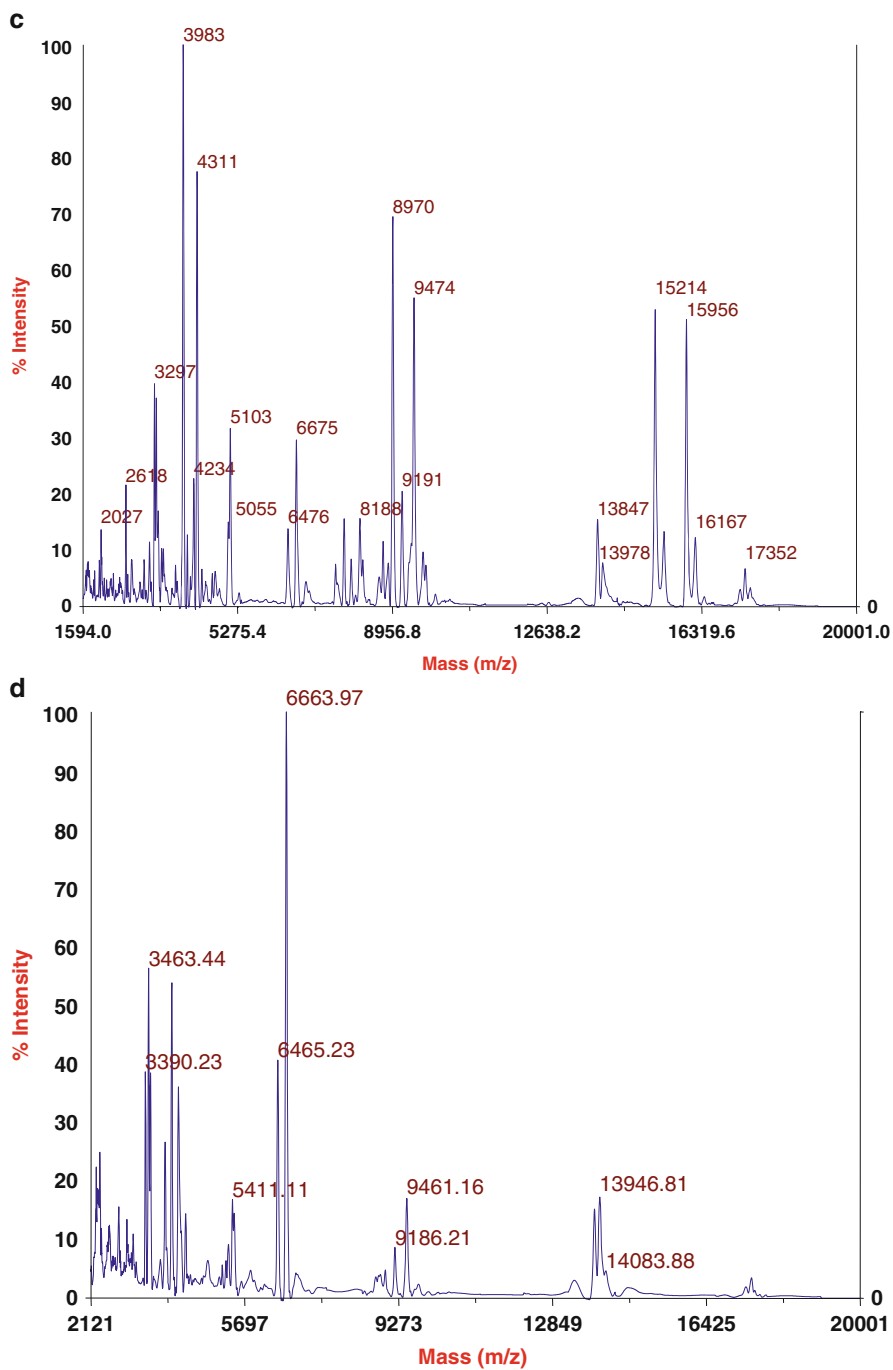


Fig. 2.2 (continued)

peptide profiles, numbering in the hundreds with optimal signal-to-noise ratios being derived from samples resulting from water solubilisation after organic solvent precipitation. Similarly, acetonitrile precipitation was shown to remove more than 99 % of the protein from serum and reduced the presence of albumin in serum from being the most abundant protein to being the 20th most abundant [43]. Further treatment of the precipitated serum sample by acid hydrolysis has also been shown to improve detection of major serum peptides (transferrin and fibrinogen) as well as other peptide species between 4 and 10 kDa [51]. Other approaches involve preparation of the sample when applied to the sample plate and alternative ionisation means other than MALDI being applied. One such method describes nanostructure initiator mass spectrometry (NIMS) in which cysteine containing peptides are captured by a maleimide chemistry and ionised without the need for a matrix to be applied, thereby reducing background noise signals and improving sensitivity [50]. An alternative, but related, approach to MALDI protein profiling is surface-enhanced laser desorption ionisation (SELDI) mass spectrometry. In SELDI preparation protocols, the biofluid in question is applied directly to the SELDI plate (equivalent to the MALDI sample plate) which has a surface chemistry similar to that found on magnetic beads and microscale tips as described previously. The sample is then washed on plate removing salts and unbound proteins and the remaining proteins analysed and presented either as raw spectra data or as interpreted gel-like images prior to statistical analysis. SELDI is most commonly utilised to identify differential protein peaks as prospective biomarkers; however, it has also been applied to the validation of previously determined HPLC–mass spectrometry results as a rapid validation method for studying levels of specific protein biomarkers in breathe condensate as indicators of chronic obstructive pulmonary disease [24]. Furthermore, SELDI has been applied in tandem with magnetic bead sample preparation utilising serum as the starting biofluid to improve peptide/protein detection [76] and advances in statistical data analysis have attempted to address some determined issues of reproducibility and accurate biomarker identification when using SELDI analysis [18].

2.3 Protein Profiling and MALDI Imaging

After sample preparation, a common application of protein profiling from biofluids is the characterisation of peptide and protein profiles followed by statistical examination of the profiles in order to determine any potentially diagnostic differences between different cohorts that might represent biomarkers of any condition under study. The analysis itself does not usually allow for the identification of the protein from which any diagnostic protein or peptide peaks arise and so only the m/z ratio of any prospective biomarker is determined by the analysis. Despite this, protein profiling of biofluids has been applied successfully in a wide range of biomedical areas. The approach has been applied widely in cancer biomarker research and protein/peptide peaks have been identified which allow for the discrimination of patients from control cohorts but also differentiate pre- and post-operative patients

and those at risk of metastasis and reoccurrence [88]. Furthermore, MALDI protein profiling of urine of patients allowed for the identification of proteins whose urinary expression could be used to identify patients at risk of liver injury due to chemotherapeutic treatments such as methotrexate [86]. Another area of well-studied interest is the application of such protocols in diabetes and kidney-related disorders. Magnetic bead preparation and MALDI analysis of urine samples were successfully shown to be able to identify three proteins/peptides with reduced expression in type two diabetes patients and further mass spectrometric analysis allowed these to be identified as three proteins without a known previous association with the disorder [13]. Peptide profiling was also applied to patients with primary nephrotic syndrome and relevant control groups and a group of 14 peptide ions were shown to have good diagnostic value offering the potential of replacing a time consuming and invasive biopsy procedure for diagnosis [34]. The analysis has also been utilised in order to further understand the potential beneficial effects of drug regimes utilised after kidney transplant. The effect of the drug paricalcitol on the serum protein profile was determined and the protein expression changes suggested to represent alterations in the expression levels of parathyroid hormone, alkaline phosphatase, bradykinin and complement factor C4, thereby suggesting secondary effects of the drugs administration [64]. More recently, protein and peptide profiling by MALDI mass spectrometry has also been applied to conditions for which the biological cause is less certain and/or variable in different cases. One key example of this is the application of the technique in psychiatric disorder diagnosis. The principle of applying different approaches to the preparation of the biofluid has been utilised predominantly to study serum protein and peptide changes. One study utilised acid hydrolysis in order to allow for peptide profiling to be undertaken in depression patients and control subjects whilst others have utilised both magnetic bead and microscale tip preparations for the same purpose [1, 35, 51]. Both allowed for the identification of different prospective biomarkers for depression with significant area under the curve values determined after the creation of receptor operator characteristic (ROC) curves. The latter analysis identified three peptide ions which had diagnostic value and whilst the accumulated relative abundance of the three biomarkers did not further improve diagnostic ability, the combined study of all three with alterations in two of the three being used as a diagnostic tool significantly improved diagnostic accuracy. This finding demonstrates the potential benefit of the MALDI peptide/protein profiling approach over techniques such as ELISAs which can only determine the expression of a single protein. The collation of the entire protein profile in a single analysis offers the utilisation of the expression of multiple species within the profile to be utilised without further analysis being undertaken and therefore various expression levels can be studied and ratios of individual proteins/peptides also utilised. Protein profiling after magnetic bead sample preparation has also been utilised in other psychiatric disorders; the technique was utilised for the analysis of serum samples from adolescent patients with autism spectrum disorder (ASD) with and without co-morbid attention deficit hyperactivity disorder (ADHD) compared to control subjects. Protein signatures were identified which differentiated all patients from control subjects and also could differentiate the ASD patients into two

groups (with and without co-morbid ADHD) [82]. A recent study considered the differentiation of obese subjects compared to control subjects utilising ultrafiltration to remove high abundance proteins and identified peptide signatures which differentiated not only patients from control subjects but also patients with and without diabetic symptoms. Such analyses suggest that the process of an unbiased protein profiling experiment can allow for the discrimination of patients with disorders whose biological cause is less certain and also allow for the differentiation of similar cohorts with different disorder diagnoses.

Whilst the majority of analyses in biomedical research utilising MALDI protein and peptide profiling have studied biofluids after sample preparation of one form or other, some have studied biological tissues and utilised a process of MALDI imaging mass spectrometry for spatial analysis of protein and peptide signals. The process of MALDI imaging requires spectra to be taken from tissues applied to a MALDI sample plate after the incorporation of a suitable matrix. Multiple spectra are obtained with high precision across the tissue and the data collated in order to demonstrate the distribution of proteins and peptides within different cells within the tissue examined. Such analysis has been utilised to study protein/peptide differences in cancer tumour cells compared to adjacent “normal” cells from formalin-fixed tissue samples and identified specific signatures associated with early stage disease and tumours with metastatic potential [80]. The analysis has also been used to determine the altered regulation of candidate proteins involved in cancer progression identified by other studies with a resolution of 30 μm [52]. Whilst cellular proteins can be easily determined by the imaging MALDI approach, it has been reported that membrane proteins are less well represented by such analyses and one study utilised a pretreatment approach to overcome this issue, allowing for the study of two known membrane proteins and their acylation status within tissues [60]. Whilst most imaging MALDI analysis allows for a single matrix to be applied and therefore one set of data to be obtained a recent approach utilised a frozen mouse brain section to be analysed with two matrices—one utilised to study proteins/peptides and a second used to emphasise lipidomic data—thereby allowing multiple analyses to be undertaken and more information to be extracted from a single tissue subsection [74]. Protein profiling from both biofluids and tissues therefore allows for diagnostic differences to be determined and whilst the biological identity of the protein or peptide in question is not obtained from the data, the unbiased analysis of profiles allows for novel biomarkers to be identified within biomedicine.

2.4 Post-translational Modification Analysis

A further application of MALDI protein and peptide profiling is in the analysis of the post-translational modification status of either the entire proteome or selected proteins or peptides. Of the available post-translational modifications, the two which have shown the greatest application to MALDI profiling are glycosylation and phosphorylation; however, the latter of these is more commonly studied utilising

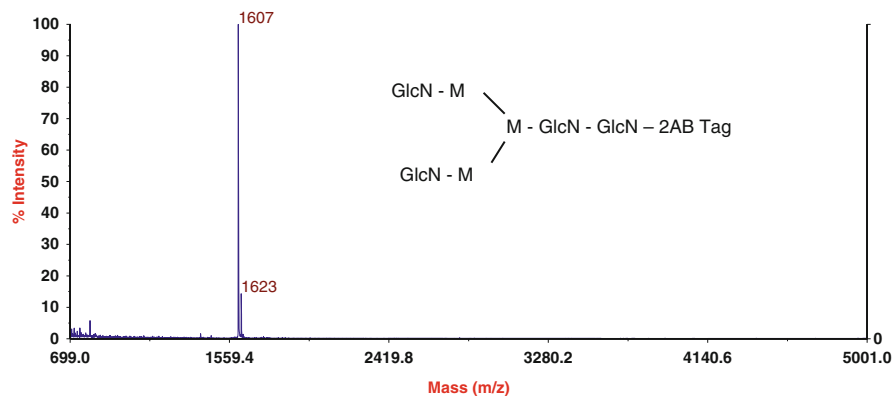


Fig. 2.3 MALDI analysis of an AB-modified glycan

HPLC–mass spectrometric methods and selective tandem mass spectrometry analysis such as constant neural loss and precursor ion discovery. Glycosylation therefore has shown the greatest application with regard to MALDI protein and peptide profiling, with the availability of magnetic bead technologies with immobilised lectin chemistries in order to allow for the selective capture of glycoproteins and glycopeptides prior to MALDI mass spectrometric analysis. The glycans themselves can be analysed after derivatisation and release from the peptide with dihydroxybenzoic acid being utilised as a matrix as shown in Fig. 2.3. Further advances in the pre-analysis preparation of glycoproteins/peptides has also encompassed the utilisation of a microscale tip purification approach which uses a 50:50 mixture of graphite and activated graphite (termed GA) in order to enrich for glycopeptides from protein digests and the utilisation of oxidised ordered mesoporous carbon as a pre-analysis purification approach [79, 95]. Larger scale pre-purification of glycoproteins commonly utilises offline separation techniques such as high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) followed by fraction collection and further MALDI mass spectrometric analysis [40]. The matrix choice when analysing glycoproteins and peptides has also previously been studied and 4-chloro-cinnamic acid suggested as a matrix for the analysis of labile groups in negative ionisation mode and a combination of CHCA and 3-aminoquinoline as a matrix applicable to complex mixture analysis when studying glycoproteome samples [68, 91]. For comparative analysis of glycoproteins between different samples a simple derivatisation method has also been developed in which glycoproteins are reacted with differently labelled anthranilic acid and then mixed, digested and analysed by MALDI profiling (in a similar manner to ITRAQ technology) with sub-picomolar limits of detection [83]. Furthermore, comparative analysis of the glycosylation modification structure itself is also possible either by separation from its proteins/peptides and chemical reaction or via in-source decay processes being utilised during the MALDI process [31, 57]. The differential glycosylation has been studied in a number of diseases, including congenital disorders specifically of glycosylation and ovarian cancer [7, 94]. In the ovarian cancer study, the

glycans themselves were cleaved from the glycoproteins and analysed by MALDI profiling and these profiles exhibited improved diagnostic potential when compared to the commonly utilised CA-125 biomarker. Further applications have also been reported for the MALDI analysis of glycoproteins within a biomedical context. The glycosylation status of proteins from stem cells during differentiation to adipose tissue (adipogenic differentiation) was studied and specific changes in the glycoproteome identified which accurately allowed the differentiation event to be monitored within the cells [30]. Other studies have focussed upon different glycosylation forms of specific proteins of interest. The application of high accurate mass MALDI profiling after purification allowed for the identification of six new apolipoprotein CIII isoforms and their glycosylation status which may have a role in lipid metabolism and transport within the body and ApoCIII isoforms have been identified as easy to purify by microscale tip preparation and able to allow for diagnoses in chronic hepatitis C and alcoholic liver cirrhosis [61]. A different approach sought to study the glycosylation status of membrane-type 1 matrix metalloproteinase (MT1-MMP) via MALDI mass spectrometric analysis after immunoprecipitation from cell lines as the glycosylation is required for cancer cell invasive processes [81]. The glycosylation status of haemoglobin has also been undertaken by MALDI profiling protocols encompassing the study of the non-enzymatic glycosylation as a result of diabetes and also differential glycosylation of different isoforms of haemoglobin suggesting that the isoform presented by individuals may act as a risk factor for glycation occurring in prospective patients of diabetes [15, 47].

The second commonly analysed post-translational modification of proteins utilising MALDI protein/peptide profiling is phosphorylation status. Numerous approaches to MALDI matrix preparation and application have been suggested as enhancing phosphopeptide profiling. Combining the matrix of 2,6-dihydroxyacetophenone (DHAP) or 2',4',6'-trihydroxyacetophenone (THAP) with the additive diammonium hydrogen citrate (DAHC) has been investigated as to their potential to improve phosphopeptide analysis and suggested less suppression by non-phosphorylated peptides during profiling without selective purification of phosphopeptides [33, 96]. However different groups have reported improved signals utilising a further matrix (dihydroxybenzoic acid) in combination with phosphoric acid as a matrix additive or combinations of two matrices (CHCA and 3-hydroxypicolinic acid (3-HPA)) [45, 100]. Further approaches to enhance phosphoprotein and peptide analysis by MALDI profiling have focussed on chemically altering the MALDI plate in order to selectively trap phosphate containing species and these have mainly utilised titanium dioxide as the linker to trap the phosphate group [89]. Phosphopeptide capture has been achieved on a sintered material before elution and subsequent MALDI profiling analysis and also by on-plate capture using photocatalytically patterned titanium dioxide distributions on plate using nanoparticles [20, 77]. Each of these utilises metal affinity to enhance the amount of phosphate containing peptide or protein in the sample prior to analysis and has been demonstrated to enhance coverage of expected phosphopeptides within tryptic digests of known phosphoproteins. Commonly, casein has been utilised as a test protein in the purification of phosphorylated peptides as it provides a known phosphopeptide in order to gauge the success of the purification technique (Fig. 2.4).

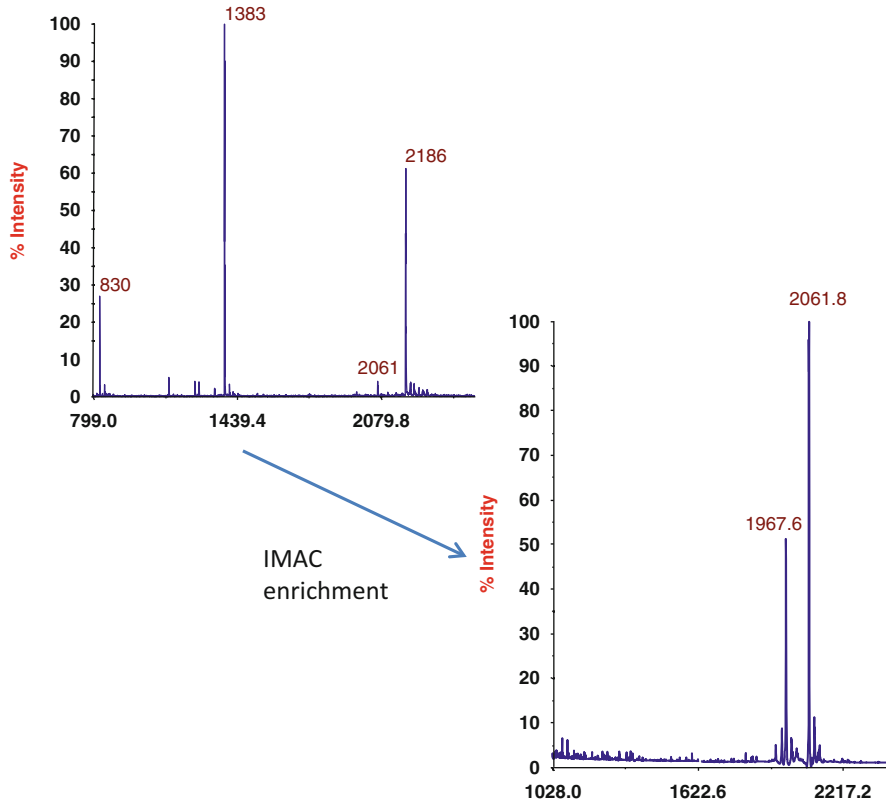


Fig. 2.4 MALDI spectra of tryptic peptides from casein before and after IMAC enrichment of phosphopeptides

2.5 Specific Protein Analysis

As well as protein profiling of entire proteomes, MALDI profiling has also been utilised in order to assay for specific proteins and variations in specific proteins in relation to different diseases. The approach has been applied to the study of diagnostic protein signatures for type 1 dengue virus, involving immunoaffinity capture on magnetic beads and analysis of the proteome by MALDI profiling allowing for differentiation of the virus from other similar viruses [10, 11]. Amyloid beta peptides have also been immunopurified (from cerebrospinal fluid) and the different isoforms studied by SELDI profiling allowing for the differentiation of 15 isoforms of the peptide of which the expression levels of many were indicative of cognitive decline in many age-related mental deterioration conditions [2]. MALDI profiling in combination with immunopurification has also been utilised to study the status of the circulating hormone Ghrelin [29]. The hormone can be acetylated and exists therefore in multiple forms and these could be studied and relative ratios determined by MALDI profiling following immunoprecipitation. In another study the

phosphorylation status of a specific protein (a target for protein kinase C) was studied from serum samples from mice with and without tumours utilising MALDI analysis and shown to provide diagnostic potential in relation to the ratio of phosphorylated and unphosphorylated peptide [41]. As well as studying natural protein modifications by MALDI profiling, the formation of organophosphorus adducts of human butyrylcholinesterase was also undertaken by MALDI analysis [37]. After exposure to the pollutant, a protein extraction was utilised and proteins digested, purified by titanium dioxide and analysed. The enzyme has the pollutant attached to it at a specific serine residue within its structure and therefore the bound and unbound mass of the tryptic peptide can be analysed by MALDI analysis as a ratio and an indicator of exposure to the pollutant.

2.6 Organism Identification

A further area in which MALDI protein profiling has been successfully applied is in the identification of organisms, primarily micro-organisms. In relation to micro-organism analysis, the protocol usually requires a sample of the micro-organism to be added to either a solvent or matrix solution. This is then added to the MALDI sample plate with additional matrix, allowed to dry and the MALDI process allows for the production of a protein profile from the organism itself. A number of groups and mass spectrometer manufacturers have developed databases of the protein profiles exhibited by known organisms and the protein profile of an unknown organism can therefore be compared to the database and an identification suggested as a result [16]. The approach has been reported as successfully identifying characteristics such as resistance in species and strain level identification within species as well as differentiating different species based on the protein profile obtained. Figure 2.5 shows spectra generated from the comparative analysis of four species of bacteria after simple plating on the MALDI plate with the addition of sinapic acid as a matrix. The process has been applied to various bacterial species including those responsible for acne, in which the differentiation of different phylotypes was also shown to be possible [59]. The method of sample preparation—in relation to both the culturing of the bacterial species and the subsequent preparation of the sample for MALDI analysis—has been investigated with broth media growth followed by a protein extraction method before analysis being reported as being optimal [27]. One group suggested that ribosomal proteins accounted for 10 of 13 species-specific peaks obtained by MALDI protein profiling and even strain-specific differences, confirming the expected mass shift by genome sequencing the genes responsible for the proteins themselves and identifying the amino acid differences resulting in the mass shifts exhibited [75]. A further study utilised a similar approach in order to confirm the identity of the peaks responsible for the differentiation of clonal strains of *Staphylococcus aureus* utilising sequencing and anti-sense RNA knockdown experiments in order to confirm the peptides responsible [38]. MALDI has also been shown to be able to differentiate enteropathogenic and

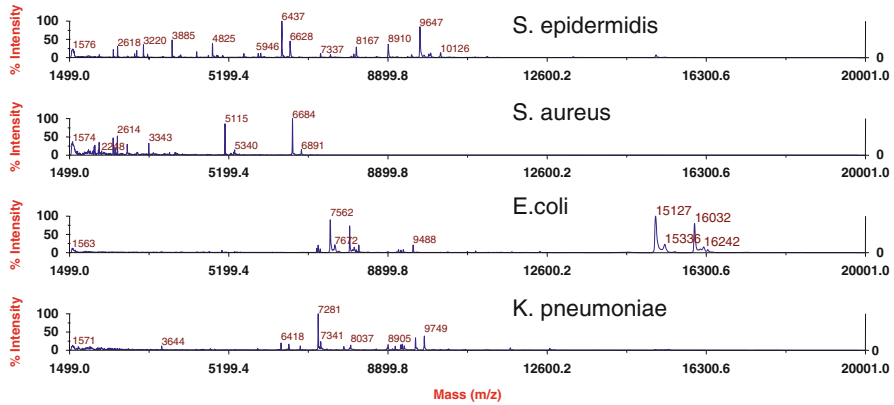


Fig. 2.5 MALDI protein profiles of four different bacteria

non-enteropathogenic strains of bacteria suggesting a role in fine-tuning the treatment of patients with bacterial infections [69]. Other studies have utilised blood culture bottles as the source of the micro-organisms under study and one study developed an optimised spin lysis/formic acid extraction method for the subsequent analysis [28, 49, 55, 71]. Such analyses would have a significant benefit to patients suffering from sepsis. However, a separate report identified a direct transfer of the sample to the plate and formic acid sample preparation as being optimal for subsequent analysis. The analysis was shown to be possible and both analysis from solid colonies and from blood cultures have been suggested to provide improvements in patient management in a hospital setting, being superior to existing blood culture methods and reducing analysis time [72].

The MALDI protein profiling approach has also been utilised to characterise rarer pathogenic bacteria and it was noted that of the various organisms studied fewer bacteria needed a second confirmation analysis when using MALDI compared to conventional approaches (50 compared to 620) [73]. Besides its application in identifying clinical bacterial infections, the MALDI protein profiling approach has also been reported in being able to confirm the identification of *Bacillus anthracis* spores in suspicious powder in less than half an hour [17]. Due to the promise suggested by such laboratory investigations inter-laboratory comparisons have been performed in order to show the reproducibility of the technique between centres, to compare competing MALDI mass spectrometers and commercial databases available and to compare the technique to existing methods of identification [23, 36, 42, 48, 53, 66, 93]. The method has also been applied to the identification of medically important fungal species such as *Candida*, allowing for phenotypical differences to be determined in 1,383 isolates examined and also for antifungal susceptibility to be accurately determined, thereby allowing for a more informed treatment of patients [46, 87]. Beyond micro-organisms, the MALDI protein profiling approach has more recently been applied to other organisms. The identification of Tsetse (*Glossina* spp.) which are vectors of the so-called sleeping sickness was undertaken after formic

acid/acetonitrile extraction from the insects allowing for species identification but not sex determination [32]. Furthermore, a similar approach was used in the study of *Anopheles* mosquito species in order to accurately differentiate between differing subspecies and was shown to be an improved approach compared to the traditional polymerase chain reaction (PCR)-based techniques commonly applied [58]. MALDI analysis has also been utilised to study and characterise venoms from different species, mainly focused on scorpions. The so-called venom mass fingerprinting (VMF) was developed to study low levels of venom (nanograms of starting material) and specific toxin components, such as short chain peptides which act on potassium channels could be easily identified within venoms [54, 65].

2.7 Single-Nucleotide Polymorphism Analysis

Whilst a major focus of MALDI profiling has been concerned with the analysis of proteins and peptides as biomolecular species, the ability of MALDI to provide good mass accuracy signals for other biomolecules at high molecular weight has also been studied. One of the major focuses of this has been in the analysis of short sequence nucleotides, especially when applied to the analysis of single-nucleotide polymorphisms (SNPs). SNPs are variations in genetic sequence in specific genes which give rise to phenotypical variations in humans and can include susceptibility to diseases and response to specific treatments. They are therefore useful as indicators of disease risk and in the development of the most appropriate treatment regimes for patients. For SNP analysis, DNA is taken from an individual and the PCR used to amplify the DNA to a sufficient quantity. A primer is then designed in order to reproduce the gene but lacking one of the four required bases for nucleotide polymer extension. The length of the extended replication product of the gene is therefore dependent upon how soon the replication process requires the lacking nucleobase in order to further extend the replicating DNA. An example is shown in Fig. 2.6. With careful primer design and knowing the expected SNP site this can allow for differing length (and as a result differing mass) of the extended gene product dependent upon (a) whether the SNP is absent from both gene copies, (b) whether the SNP is present in only one gene copy and (c) whether it is present in both gene copies. Given that the expected resulting mass shift is usually a few hundred mass units, this can be clearly detected by the MALDI approach in sequences up to 20–30 nucleotides in length. Furthermore, different genes and different SNPs can be analysed simultaneously as long as the mass of the primer sequences and their extension products is sufficiently different to be determined separately by the MALDI profiling process. The approach has been applied successfully in a number of different disorders including cancer, lupus, arthritis, cirrhosis, insulin sensitivity and cholesterol metabolism disorders [10, 11, 14, 39, 63, 70, 97-99]. Usually after extension, the oligonucleotides are desalted using cation exchange beads (due to the fact that the PCR requires the addition of magnesium chloride which then interferes with the MALDI process) and mixing with a hydroxypropylonic acid matrix with ammonium citrate as an additive.

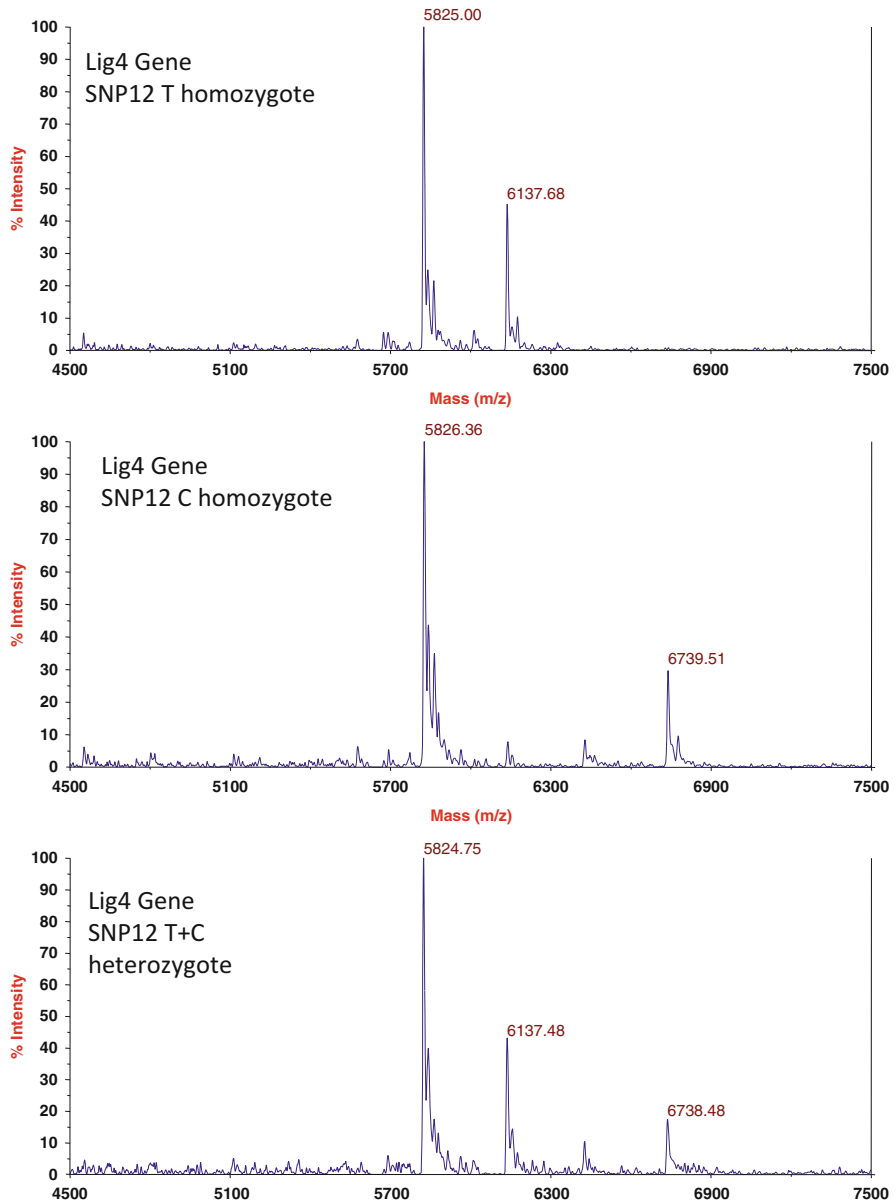


Fig. 2.6 Single-nucleotide polymorphism analysis utilising MALDI mass spectrometry

2.8 Metabolomics

Metabolomic analysis does not commonly utilise the MALDI approach due to matrix ions in the low mass range (less than 500 Da) which complicate the resulting

spectra. However, more recently, the approach has been utilised to study cell metabolism in cancer cells, metabolites from blood spots and amino acids present in the natural secretions of *Lucilia sericata* (the larvae of the greenbottle fly, utilised in maggot debridement therapy) [6, 44, 62]. Of the study of different metabolite profiles, the most common application of MALDI profiling is in the field of lipidomics in which phospholipids and associated species can be studied in a high-throughput manner using the mass accuracy provided by the ToF tube to allow for the grouping of phospholipids by head group and the revealing of the total length of both fatty acid chains combined and the total number of double bonds presented by both fatty acids combined. Usually, phosphatidylcholine lipids are analysed in positive ionisation mode due to the easily protonated head group associated with this class whilst the other lipid groups provide an improved signal in negative ionisation mode. The matrices utilised for the two ionisation modes differ, with dihydroxybenzoic acid in trifluoroacetic acid and methanol allowing for positive ionisation and *p*-nitroalanine in 2:1 chloroform:methanol being utilised for negative phospholipid analysis. Example spectra are shown in Fig. 2.7, alongside the effect of dosing the matrix with caesium chloride prior to analysis (causing a mass shift of 132 amu) which allows for the differentiation of different lipids and their sodium adducts in positive ionisation analyses. MALDI profiling in phospholipid analysis has found applications in the study of the species in eye lens, the lipid profiling of cancer cells, studies as to embryo optimisation and studying changes in the biochemistry of spermatozoa in obese patients [19, 44, 67]. In order to improve such analysis polystyrene spheres have been suggested as a matrix additive in order to improve matrix heterogeneity during crystallisation and precoating the MALDI plate with 1,5-diaminonaphthalene has been used in order to allow MALDI imaging of phospholipid distributions [92, 97]. Therefore, it can be seen that metabolite profiling, especially subclasses of metabolites such as phospholipid profiling, is rapidly improving based on advances already applied to protein and peptide profiling.

2.9 Conclusion

MALDI as a technique first found a niche role in allowing for the mass spectrometric analysis of large biomolecules (proteins predominantly) as a singly charged species. However it has been recognised as a potentially high-throughput method for the analysis of a variety of other biochemical entities. With improvements in matrix choices, heterogeneity of matrix crystallisation with analytes and post-acquisition data analysis tools, MALDI has found applications in studying both the presence and, in MALDI imaging, the distribution of many more biological moieties and applications in low molecular weight metabolites are also increasing in their scope and applications.

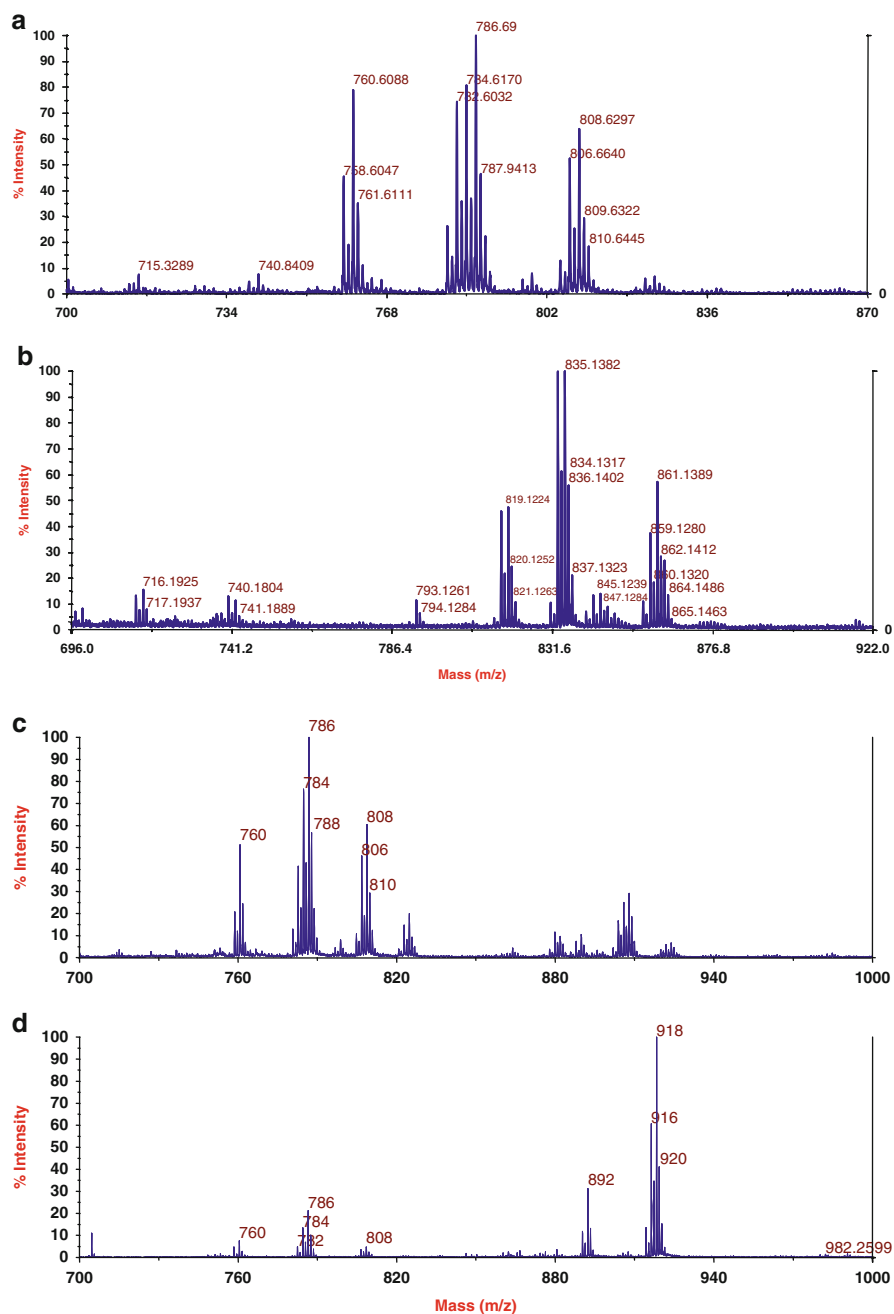


Fig. 2.7 Analysis of phospholipids by MALDI mass spectrometry. (a) Positive ionisation analysis. (b) Negative ionisation analysis. (c) Positive ionisation analysis without caesium chloride. (d) Positive ionisation analysis with caesium chloride

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