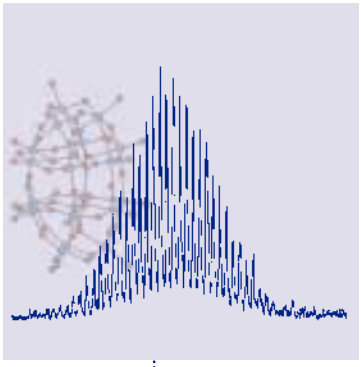
6 · 2001

Analytix

Advances in Analytical Chemistry

SIGMA-ALDRICH

MALDI-Mass Spectrometry



MALDI (matrix-assisted laser desorption/ionization), a laser-based soft ionization method has proven to be one of the most successful ionization methods for mass spectrometric analysis and investigation of large molecules. Developed in the late 1980s [1, 2] from similar desorption/ionization mass spectrometric methods such as FAB (fast atom bombardment) and LDMS (laser desorption mass spectrometry), its distinguishing feature is that the sample is embedded in a chemical matrix (ca. 1000x molar excess) that greatly facilitates the production of intact gas-phase ions from large, nonvolatile, and thermally labile compounds such as proteins, oligonucleotides, synthetic polymers and large inorganic compounds. A laser beam (UV- or IR-pulsed laser) serves as the desorption and ionization source. The matrix plays a key role in this technique by absorbing the laser light energy and causing a small part of the target substrate to vaporize. Once the sample molecules are vaporized and ionized they are transferred electrostatically into a mass spectrometer where they are separated from the matrix ions and individually detected, usually by TOF (time-of-flight) mass spectrometry. Figure 1 shows the homogeneity of a protein labeling reaction (1.4-fold molar excess; matrix: sinapic acid) being easily monitored by MALDI-MS. The label ratio is indicated over the peaks of the different conjugates [3].
Analysis by MALDI mass spectrometry may be divided into two steps.

The first step involves preparing a sample by mixing the analyte with a molar excess of matrix. The typical matrix for use with ultraviolet lasers is an aromatic acid with a chromophore that strongly absorbs the laser wavelength. Other laser wavelengths are possible, in particular the mid-infrared range where the matrix can be energized by vibrational excitation; different matrix compounds must be used in this case.
The second step of the MALDI process involves desorption of bulk portions of the solid sample by a short pulse of laser light.

MALDI Matrices: Properties and Requirements

The MALDI matrix must meet a number of requirements simultaneously:

- be able to embed and isolate analytes (e.g., by co-crystallization)
- be soluble in solvents compatible with analyte
- be vacuum stable
- absorb the laser wavelength
- cause co-desorption of the analyte upon laser irradiation
- promote analyte ionization

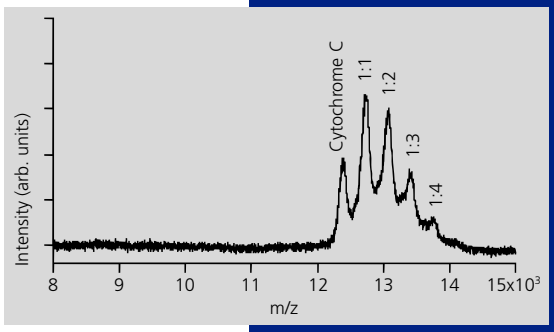


Figure 1:
MALDI TOF mass spectra of Cytochrome C covalently labeled by biotin-NHS (biotinamidocaproate N-hydroxysuccinimide ester)

Contents:

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It is believed that compounds with labile protons, such as carboxylic acids, are good MALDI matrices in the positive ion mode because they are easily able to protonate neutral analyte molecules in the plume. However, an acidic environment is not always desirable, in particular if denaturation of the tertiary structure of biomolecules should be avoided. Therefore mostly nonacidic matrices are used for protein measurements [4]. Compounds that are not easily protonated can be cationized instead, often by adding a small quantity of salt to the sample (alkali cations, and also Cu or Ag). It is also possible to detect analytes as radical cations by employing so-called electron transfer matrices [5]. More easily deprotonated compounds, such as oligonucleotides, are usually detected in negative ion mode.
The MALDI method has been developed empirically and despite its widespread use, the factors that determine success or failure of MALDI experiments are not yet fully understood. Investigations of the MALDI mechanism have recently become the focus of interest of a number of research groups [6].

Sample Preparation Techniques

In its current state, MALDI is primarily based on the laser desorption of solid matrix-analyte deposits [7]. The technique suffers from some disadvantages such as low shot-to-shot reproducibility, short sample life time and strong dependence on the sample preparation method, figure 2. A few research groups have investigated the use of liquid matrices [8], to increase sample lifetime and eliminate the search for sweet-spots, by exploiting the self-healing properties of the sampling position through molecular diffusion.

Dried-Droplet

This is the original simple sample preparation procedure introduced in 1988 by Hillenkamp and Karas [9], which has remained, with minor modifications, intact for over a decade. A drop of aqueous matrix compound solution is mixed with analyte solution and dried, resulting in a solid deposit of analyte-doped matrix crystal that is introduced into the mass spectrometer for analysis. The analyte/matrix crystals may be washed to remove the non-volatile components of the original solution. This method tolerates the presence of salts and buffers very well, but has its limits. It is usually a good choice for samples containing more than one protein or peptide component.

Vacuum-Drying

The vacuum-drying crystallization method is a variation of the dried-droplet method in which the final analyte/matrix drop applied to the sample stage is rapidly dried in a vacuum chamber. The vacuum-drying helps to reduce the size of the analyte/matrix crystals and increases crystal homogeneity. It greatly improves spot-to-spot reproducibility and minimizes the need to search for sweet spots. The formation of smaller crystals offers the added advantage of thinner samples and improved mass accuracy and resolution. Peptides and proteins analyzed with the vacuum-drying method tend to exhibit extensive alkali cation adduction. This can be substantially reduced by washing the crystals directly on the probe with cold water.

Crushed-Crystal

The crushed-crystal method was specifically developed to allow for the growth of analyte doped matrix crystals in the presence of high concentrations of non-volatile solvents (i.e. glycerol, 6M urea, DMSO, etc.) without any purification. The films produced are more uniform than dried-droplet deposits, with respect to ion production and spot-to-spot reproducibility. The disadvantages of the crushed-crystal method are the increase in sample preparation time caused by the additional steps. It requires strict particulate control during solution preparation to eliminate the presence of undissolved matrix crystals that can shift the nucleation from the metal surface to the bulk of the droplet.

Fast-Evaporation

The fast-evaporation method was introduced with the main goal of improving the resolution and mass accuracy of MALDI measurements. Matrix and sample are handled separately. A drop of the matrix solution is applied to the sample stage and the solvent is allowed to evaporate. On top of the matrix a drop of the analyte solution is applied and allow to dry. After the drop has dried it is introduced into the mass spectrometer for analysis. The process delivers stable and long lived matrix films that can be used to precoat MALDI targets.

Overlayer

The overlayer method combines features of the crushed-crystal method and the fast-evaporation method. It involves the use of fast solvent evaporation to form the first layer of small crystals, followed by deposition of a mixture of matrix and analyte solution on top of the crystal layer. The difference between the fast evaporation and the overlayer method is in the second-layer solution. The addition of matrix to the second step is be-

Solid matrix	Liquid matrix	Special preparations
Dried-droplet	Chemical liquid	Solid supports
Vacuum-drying	Particle-doped (two-phase) liquid	MALDI on 2D-gels
Crushed-crystal	Chemical-doped liquid	Insoluble samples
Fast-evaporation		
Overlayer		
Sandwich		
Spin-coating		
Slow-crystallization		
Electrospray		
Quick & dirty		
Matrix-precoated targets		

Figure 2:
Different sample preparation methods

lieved to provide improved results, particularly for proteins and mixtures of peptides and proteins.

Sandwich

The sandwich method is derived from the fast-evaporation method and the overlayer method. In this method the sample analyte is not premixed with matrix. A sample droplet is applied on top of a fast-evaporated matrix-only bed as in the fast-evaporation method, followed by the deposition of a second layer of matrix in a traditional (non-volatile) solvent. The sample is basically sandwiched between the two matrix layers. It was used first for the analysis of single mammalian cell lysates by mass spectrometry.

Spin-Coating

The preparation of near homogeneous samples of large biomolecules, based on the method of spin-coating sample substrates was reported for the first time by Perera and colaborators [10]. In the original report, large volumes (3–10 µl) of the premixed sample solution were deposited on 1" diameter stainless steel and quartz plates. The samples were very homogeneous and generated highly reproducible and much enhanced molecular-ion yields from all regions of the sample target.

Electrospray

A small amount of matrix-analyte mixture is electrosprayed from a HV-biased (3–5 KV) stainless steel or glass capillary onto a grounded metal sample plate, mounted 0.5–3 cm away from the tip of the capillary. Electrospray sample deposition creates a homogenous layer of equally sized microcrystals and the guest molecules are evenly distributed in the sample. The method has been proposed to achieve fast-evaporation and to effectively minimize sample segregation effects.

Quick & Dirty

The quick & dirty (Q&D) sample preparation separates matrix handling from sample handling. In most cases very little or no sample purification is needed prior to the analysis. A drop of matrix solution is added on top of a drop of analyte solution (0.1–10 mM). Both solutions are mixed thoroughly with the pipette tip before the mixture is dried under an air or nitrogen stream). The sample is then introduced into the mass spectrometer.

The advantages of the Q&D method are multiple:

- It is fast. It separates the sample and matrix preparation steps.
- It can be used for the analysis of in-plate protein digestions.
- It makes it very easy to add a calibration standard to the sample.

The main disadvantage of the method is that it provides the least control of all sample preparation procedures.

Matrix-precoated Layers

This sample preparation method is reduced to the straightforward addition of a single drop of undiluted sample to a precoated target spot. The advantages are: faster preparation and more sensitive results than previously described methods. It also offers the opportunity to directly interface the MALDI sample preparation to the output of LC and CE columns. Most efforts have focused on the development of thin-layer matrix-precoated membranes e.g. nylon, PVDF, nitrocellulose, anion- and cation-modified cellulose, regenerated cellulose or regenerated cellulose dialysis membrane.

Chemical Liquid

The preparation of such samples is rather straightforward. An appropriate molar ratio of the analyte is dissolved in the liquid matrix, often by employing a solvent that is evaporated before introduction of the sample into the vacuum system of the mass spectrometer.

Particle-doped (two-phase) Liquid

In the case of two-phase matrices, a suspension of particles (fine metal or graphite with a diameter of 1µm or less) in a solvent is mixed with analyte and some binder. The solvent is evaporated and the remaining "paste" on the sample holder is introduced in the ion source. Care should be taken about possible contamination of vacuum pumps and ion optical elements by small particulates that are sputtered from such samples. The fine particles absorb most of the energy from the laser beam and promote the desorption. The liquid molecules provide the charge for ionization. Several combinations of particulates and liquids have been used to analyze proteins, oligosaccharides, synthetic polymers, and dyes up to a molecular weight of over 10,000.

Chemical-doped Liquid

An organic compound, highly absorbent at the frequency of the laser, is added to the liquid medium. Very often the compounds used are the traditional MALDI matrix materials. The additive absorbs the energy from the laser and provides the charge during ionization.

Figure 3:
Laser sources used for MALDI

Laser	Wavelength	Photon energy (kcal/mol)	Photon energy (eV)	Pulse width
Nitrogen	337 nm	85	3.68	< 1 ns - few ns
Nd:YAG µ3	355 nm	80	3.49	typ. 5 ns
Nd:YAG µ4	266 nm	107	4.66	typ. 5 ns
Excimer (XeCl)	308 nm	93	4.02	typ. 25 ns
Excimer (KrF)	248 nm	115	5.00	typ. 25 ns
Excimer (ArF)	193 nm	148	6.42	typ. 15 ns
Er:YAG	2.94 µm	9.7	0.42	85 ns
CO_2	10.6 µm	2.7	0.12	100 ns+1 µs tail

Solid Supports

This method includes solid supports that play the role of the matrix. Best known is the use of porous Si as a sample support, without the need for any other matrix (desorption/ionization off silicon or DIOS). This is somewhat related to the particle matrices discussed above. The concept of an "active" MALDI support is very promising for practical applications; current limitations include a limited mass range, poor reproducibility of the fabrication process, and a fairly serious effect of surface contamination.

MALDI on 2D-Gels

Special protocols even allow complex samples to be studied by MALDI MS directly: detection of the proteome of intact cells, MALDI imaging of biological thin sections, or the analysis of 2D-gels not only by excising spots, but also by direct analysis of the plate come to mind.

Insoluble Samples

A special challenge is presented by samples that are insoluble. It seems impossible to embed them in a matrix environment by any of the above mentioned methods. It has been found, however, that by pressing a mixture of finely ground sample and analyte, it is possible to record MALDI data from insoluble compounds [11], for example insoluble or high molecular weight synthetic polymers. An added benefit is that rather thick samples are produced by this method, resulting in long lived samples that give signal for thousands of laser shots impinging in one location.

Ionization Sources for MALDI-Matrices

Most MALDI matrices are optimized for UV wavelengths, driven largely by the availability of economical and compact nitrogen lasers that emit at 337 nm. These lasers are found in most commercial instruments. Alternatively, frequency tripled Nd:YAG lasers at 355 nm are sometimes used. In the IR range, the most frequently used laser source is an Er:YAG laser emitting at 2.94 µm. Er:YAG lasers are quite a bit more expensive than nitrogen lasers, and instrument companies tend to only offer IR MALDI as an option. A significant advantage is that IR-MALDI is somewhat softer than UV-MALDI, for example in the analysis of oligonucleotides or noncovalently bound complexes of biomacromolecules. Disadvantages include a limited choice of good IR-MALDI matrices, the much larger penetration depth of infrared radiation into the sample, a larger depth of vaporization per shot which leads to short lifetime of the sample, and a somewhat lower sensitivity compared to UV-MALDI. Figure 3 presents a summary of laser wavelengths, pulse widths, and corresponding photon energies used for MALDI. Absorbers other than UV or IR chromophores can also be used, e.g. dye compounds absorbing in the visible spectral range.

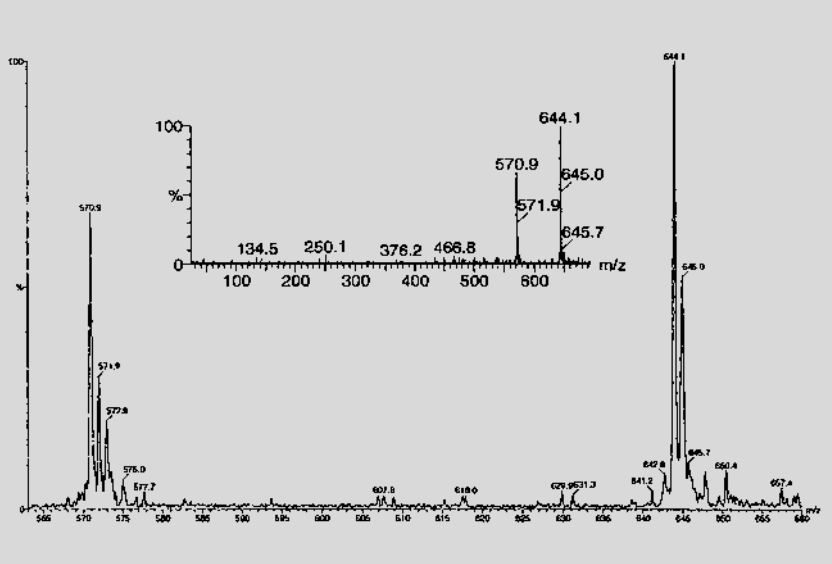

Figure 5:
Mass spectrum of the Rh-Complex

Figure 6:
Rhodium complex

Applications and Choice of Matrix

The most important applications of MALDI mass spectrometry are (in decreasing order of importance): peptides and proteins, synthetic polymers, oligonucleotides, oligosaccharides, lipids, inorganics. Numerous matrices have been found for these and other classes of compounds; a summary is given in figure 4. Although electrospray ionization (ESI) is somewhat competitive and certainly complementary, MALDI remains the method of choice in several key areas, particularly proteomics. ESI mass spectra include many peaks of multiply charged ions which can complicate the interpretation of the spectra of complex samples. Also, the ion current is distributed over a range of m/z values, sometimes compromising sensitivity. The sensitivity of ESI is also severely reduced by the presence of salts, impurities, and organic buffers which are more easily tolerated by MALDI.

MALDI-MS for Inorganics

Compared to the large number of applications for organic (especially bioorganic) compounds the use of the matrix assisted laser ionization method for the analysis of inorganic compounds is relatively rare [12]. Nevertheless there is a good chance to get useful MALDI mass spectra from many inorganic compounds with the appropriate choice of the matrix. With metal complexes one should keep in mind that the matrix may occupy a coordination site. Also the acidic nature of many matrices is destructive to proton sensitive compounds. It is best to start experiments with an aprotic matrix like T-2-[3-(4-t-Butyl-phenyl)-2-methyl-2-propenylidene]malononitrile, DCTB. The positive mass spectrum of a Rhodium complex (figure 6) preparation with DCTB matrix is shown in figure 5. The peak at m/z 644 corresponds to the $(M\text{-}Cl)^+$ whereas the m/z 571 is formed by the loss of COD. Dry droplet preparation with a compound/matrix ratio of approximately 1/20 was applied.

Figure 4:
Guide to Sample Preparation with Fluka products and Sigma Calibration Kits and Fluka Validation Sets

Determined molecule	Abbrev.	Product	Cat.No.
Peptide/protein			
Mass < 10 kDa	CHCA	α-Cyano-4-hydroxycinnamic acid	70990
Mass > 10 kDa	SA	Sinapic acid	85429
	HABA	2-(4-Hydroxyphenylazo)benzoic acid	54793
IR-Laser		Succinic acid	14078
UV-Laser		2,6-Dihydroxyacetophenone	37468
UV-Laser		Ferulic acid	46278
UV-Laser		Caffeic acid	60018
Liquid matrix		Glyerol	49771
Liquid matrix		4-Nitroaniline	72681
Oligonucleotide			
Mass < 3.5 kDa	THAP	2,4,6-Trihydroxyacetophenone	91928
Mass > 3.5 kDa	HPA	3-Hydroxypicolinic acid	56197
		Anthranilic acid	10678
		Nicotinic acid	72311
		Salicylamide	84228
Synthetic polymer			
Non-polar	IAA	Trans-3-indoleacrylic acid	57288
	DIT	Dithranol	10608
Polar	DHB	2,5-Dihydroxybenzoic acid	85707
IR-Laser		Succinic acid	14078
Organic molecules			
	DHB	2,5-Dihydroxybenzoic acid	85707
		Isovanillin	59927
Carbohydrates			
	DHB	2,5-Dihydroxybenzoic acid	85707
	CHCA	α-Cyano-4-hydroxycinnamic acid	70990
		3-Aminoquinoline	07336
Acidic	THAP	2,4,6-Trihydroxyacetophenone	91928
Lipids	DIT	Dithranol	10608
Dendrimers			
	SA	Sinapic acid	85429
	DIT	Dithranol	10608
Fullerenes	SA	Sinapic acid	85429
Inorganic molecules	DCTB	T-2-(3-(4-t-Butyl-phenyl)-2-methyl-2-propenylidene)malononitrile	87884
Oligosaccharide		1-Isoquinolinol	55433
Peptide, Proteins			
Mass range of standards:			
757 Da to 66,430 Da		ProteoMass™ Peptide&Protein Calibration Kit	MS-CAL1 (Sigma)
757 Da to 3,494 Da		ProteoMass™ Peptide Calibration Kit	MS-CAL2 (Sigma)
5,730 Da to 66,430 Da		ProteoMass™ Protein Calibration Kit	MS-CAL3 (Sigma)
M_p 700-80 000		MALDI Validation Set poly(butyl acrylate)	03596
M_p 500-20 000		MALDI Validation Set polyethylene glycol	03598
M_p 500-70 000		MALDI Validation Set poly(methyl methacryllate)	03599
M_p 500-70 000		MALDI Validation Set polystyrene	03565
M_p 5000-20 000		MALDI Validation Set PS, PMMA, PDMS, PEG, PSS	03597

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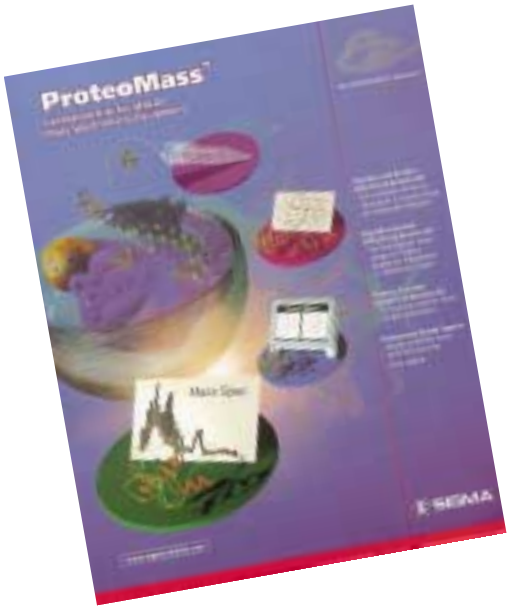

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