Basic principle: desorption of the analyte, preliminarily dissolved in a “matrix” and then co-deposited on a “target”, is achieved by a laser.

Analyte molecules (or their clusters with matrix molecules), desorbed from the surface, are ionized and then transferred to the mass analyzer.
Due to the very short times required for ion generation, a Time of Flight (ToF) analyzer, eventually equipped with one (or more) reflectron(s) is adopted:

A X-Y stage enables a controlled positioning of the target plate under the laser beam.
The target plate contains several wells, which can be filled with the matrix-analyte mixture.

The laser beam can be then directed onto specific wells (usually with the aid of a camera) and the corresponding MS spectra can be recorded.
MALDI, a simplified view of the process

(i) A ‘solid solution’ of matrix and analyte is formed on top of the target

(ii) Matrix photo-excitation
Some of the laser energy incident on the solid solution is absorbed by the matrix, causing:

✓ rapid electronic/vibrational excitation,
✓ localized disintegration of the solid solution,
✓ formation of clusters made up of a single analyte molecule surrounded by neutral and excited matrix molecules.

The matrix molecules evaporate away from these clusters to leave the excited analyte molecule:
(iii) Analyte Ionization

The analyte molecules (A) are ionized through simple protonation/cationization by the photo-excited matrix, leading to the formation of the typical [A+X]^+ type species (where X = H, Li, Na, K, etc.).

Less frequently, analyte ions are formed through deprotonation/anionization ([A-H]^- or [A+X]^- species, with X = anion).

Multiply charged species, dimers and trimers can also be formed.
Ion formation in MALDI-MS

Despite the impressive range of applications reported for MALDI-MS, the nature of the ionization process has remained poorly understood for a long time. The main reasons for such a situation are:

- no single mechanism can explain all the ions observed, even in a single MALDI experiment;

- several parameters affect the mass spectrum through the ion formation process:

  - different mass analyzers with different observation time scales
  - acceleration fields
  - sample temperature
  - incident angle of the laser beam
  - laser wavelengths
  - laser pulse energies
there are many different classes of analyte molecules, many different MALDI matrices or co-matrices, and even more matrix-analyte combinations.

the overall ion-to-neutral ratio in (UV) MALDI experiments is about $10^{-4}$ i.e. the species of interest are, by far, minoritary in the process.

The key problem in investigating MALDI is that it is a complex chemical event: one-laser-shot $\Rightarrow$ one-mass spectrum.

Despite the involvement of a very low absolute amount of material, a dense plume, containing matrix neutrals as well as reactive species, such as matrix radicals, electrons, hydrogen atoms, is formed.

Within this plume suitable analytes are ionized with high efficiency and detected with high sensitivity.
Main experimental parameters in MALDI-MS

Laser wavelength

<table>
<thead>
<tr>
<th>Laser</th>
<th>Wavelength (nm)</th>
<th>Photon energy (eV)</th>
<th>Pulse width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>337</td>
<td>3.68</td>
<td>&lt;1 ns – few ns</td>
</tr>
<tr>
<td>Nd:YAG × 3</td>
<td>355</td>
<td>3.49</td>
<td>typ. 5 ns</td>
</tr>
<tr>
<td>Nd:YAG × 4</td>
<td>266</td>
<td>4.66</td>
<td>typ. 5 ns</td>
</tr>
<tr>
<td>Excimer (XeCl)</td>
<td>308</td>
<td>4.02</td>
<td>typ. 25 ns</td>
</tr>
<tr>
<td>Excimer (KrF)</td>
<td>248</td>
<td>5.00</td>
<td>typ. 25 ns</td>
</tr>
<tr>
<td>Excimer (ArF)</td>
<td>193</td>
<td>6.42</td>
<td>typ. 15 ns</td>
</tr>
<tr>
<td>Er:YAG</td>
<td>2.94 μm</td>
<td>0.42</td>
<td>85 ns</td>
</tr>
<tr>
<td>CO₂</td>
<td>10.6 μm</td>
<td>0.12</td>
<td>100 ns + 1 μs tail</td>
</tr>
</tbody>
</table>

Nd:YAG: Neodimium-doped Yttrium-Aluminum Garnet (Nd:Y\textsubscript{3}Al\textsubscript{5}O\textsubscript{12})

Excimer (exciplex): a short-lived dimeric (heterodimeric) molecule formed from two species only when at least one is in an electronic excited state.
Laser pulse width

The laser pulse width has little or no influence on MALDI mass spectra, at least up to widths of 5-30 ns.

This result indicates that the desorption/ionization process is determined by the energy density supplied to the sample by the laser pulse (fluence, J/cm$^2$), rather than by the rate of energy flow (irradiance, W/cm$^2$).
Matrices

The choice of matrix is crucial for success in MALDI-MS experiments and for the control of fragmentation.

Depending on the matrix adopted, the matrix-sample mixtures in MALDI can be classified into three categories:

- **Solid (most common)**
  material obtained by co-crystallization of analyte and matrix

- **Liquid**
  IR or UV absorbing liquid containing the analyte

- **Two phase**
  liquid with absorbing solid (fine particles dispersed in glycerol)
Common matrices consist of small organic compounds exhibiting a strong resonance absorption at the laser wavelength used.

The matrix-effect is described to be three-fold:

1. provide a controllable energy transfer to the condensed-phase matrix-analyte mixture, inducing a uniform and soft desorption

2. promote analyte ionization by chemical reactions

3. generate favourable pre-requisites by isolating analyte molecules

MALDI matrices have also to satisfy two requirements:

✓ being soluble in the same solvent adopted for the analyte

✓ being stable under high vacuum conditions
Some representative matrices for nitrogen laser (337 nm)

2,5-Dihydroxy- benzoic acid (DHBA)
(polysaccarides)

Sinapinic acid (SA)
(proteins)

Alpha Cyano-4-hydroxy-cinnamic acid
(αCHCA)
(peptides & proteins)

3-Hydroxy-picolinic acid (3HPA)
(nucleic acids)

Unfortunately, there are still no clear guidelines for matrix selection for a particular analytical problem and better matrices are, therefore, often found only after screening a large number of candidate compounds.
The absorption (solid line) and diffuse reflection (dashed line) spectra obtained for different positional isomers of DHBA explain the effects of laser wavelength on the MALDI-MS response for a typical analyte, cytochrome C:
The type of crystals obtained will change with matrix, as obvious, but also with drying conditions and solvents adopted. In some cases, like vacuum-dried DHBA, an amorphous deposit will be obtained.
The desorption-ionization process in MALDI

Ion generation is commonly accepted to be the result of a convolution of three processes, desorption and primary/secondary ionization:

Desorption) by definition it includes the excitation of samples, the subsequent phase change and the dynamics of the material plume expansion.

Primary ions formation) the generation of the first ions from neutral molecules in the sample during the laser pulse. These ions are often matrix (M) - derived species.

Secondary ions formation) the generation of ions during subsequent matrix-matrix or matrix-analyte reactions.
The desorption process

The most common models hypothesised for the UV-MALDI desorption process are:

✓ thermal desorption of individual molecules

✓ surface layer-by-layer sublimation/evaporation

✓ volume ablation by phase explosion (also called explosive boiling)

✓ volume ablation caused by laser-induced pressure pulses.

It is likely that all the cited processes are present during a MALDI experiment, although at a different extent.

Volume ablation processes, implying expulsion of large cluster or even “chunks” of matrix/analyte material from the sample plate, are supposed to occur when high fluence (i.e. energy) lasers are employed (i.e. hundreds of J/m²).
SEM photographs taken on specific areas of matrix deposits after several laser shots, like those obtained for 2,5-DHB and α-CHCA after exposure to a N₂ laser (337 nm, pulse width 3 ns) at fluences corresponding to the ion threshold fluence, $H_0$, or higher:

suggest that melting and recondensation, leading to characteristic surface corrugations, may occur during laser irradiation.
MALDI Plume

The UV MALDI plume can be described as the result of a very rapid, even explosive, solid-to-gas phase transition, occurring within tens of ns after a ns laser pulse has hit the sample surface. Matrix and analyte molecule/clusters are projected upwards by the explosion.

As shown in a simulation of MALDI plume 1 µs after the laser impact:

Primary ions (red dots) are created in a moderately hot, dense cloud of neutral matrix molecules and clusters (blue dots):

Many of them will undergo collisions before being extracted into the drift region of the mass spectrometer.
In 1999 Puretzky and Geohegan used a gated Laser Induced Fluorescence technique to take time-resolved images of a 3-HPA MALDI plume.

MALDI desorption was induced by a KrF (excimer) laser beam with elliptical spot.

A second laser was then fired, as a sheet beam, at variable time delays after the desorption laser pulse, to stimulate fluorescence of matrix or analyte molecules.

The Laser-Induced Fluorescence (LIF) for the matrix and for a dye-tagged analyte, a large protein (Deoxyribonuclease I, 29 kDa, in a $10^{-4}$ molar ratio with respect to the matrix), was monitored, at specific wavelengths, with a Charged-Coupled Device (CCD) camera at increasing distances from the target plate.
The images obtained at increasing times show sections of the MALDI plume:

It is apparent that, within 20 $\mu$s, matrix molecules/clusters are projected upwards from the sample stage up to 2-3 cm!

At the same delay time, sample molecules are concentrated much closer to the sample stage and their spatial distribution is sharper.
The relative density of the MALDI plume can be plotted versus time:

In the expanded view, referred to the first 40 ns, the time dependence of the laser pulse (blue line) and of the distribution of matrix photo-excited states (green line) are overlapped. The dashed line refers to the density evolution observed if a phase explosion occurs.

The time ranges for primary and secondary ionizations are also evidenced.
Primary ions generation

The following mechanisms have been hypothesised for the generation of the first ions from neutral molecules in the sample during the laser pulse, i.e. before a significant MALDI plume has formed:

- Single Molecule multi-photon Ionization
- Energy Pooling
- Excited-State Proton Transfer
- Disproportionation
- Desorption of preformed ions
- Thermal ionization
Single Molecule multi-photon Ionization

According to this mechanism a matrix molecule is excited after absorbing a photon of laser radiation.

The excited state lives for a short time, some ns, and one (or more) further photons can be absorbed to yield the matrix radical cation and a free electron:

\[
\begin{array}{c}
\text{hv} \\
\text{M} \rightarrow \text{M}^* \rightarrow \text{M}^{+\cdot} + e^- \\
m = 1 \text{ (typically)}
\end{array}
\]

A two-photon second step \((m = 2, 3 \text{ photons overall})\) is unlikely, because typical MALDI irradiances, about \(10^6 - 10^7 \text{ W/cm}^2\), are too low to promote such processes.

In the simplest process \((m = 1)\), a total of two laser photons must excite the matrix molecule from the ground state to above its ionization potential (IP).
The ionization potential values for typical MALDI matrices range between 7.8 and 9.4 eV:

<table>
<thead>
<tr>
<th>Matrix</th>
<th>IP (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5 DHB</td>
<td>8.05</td>
</tr>
<tr>
<td>(2,5 DHB)$_2$</td>
<td>7.93</td>
</tr>
<tr>
<td>2,5 DHB · H$_2$O</td>
<td>7.78</td>
</tr>
<tr>
<td>5-Me-2-OH-benzoic acid</td>
<td>7.84</td>
</tr>
<tr>
<td>2,3 DHB</td>
<td>8.27</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>9.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laser</th>
<th>Photon energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>3.68</td>
</tr>
<tr>
<td>Nd:YAG × 3</td>
<td>3.49</td>
</tr>
<tr>
<td>Nd:YAG × 4</td>
<td>4.66</td>
</tr>
<tr>
<td>Excimer (XeCl)</td>
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<tr>
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</tr>
<tr>
<td>Excimer (ArF)</td>
<td>6.42</td>
</tr>
<tr>
<td>Er:YAG</td>
<td>0.42</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Such values are considerably higher than the energies related to typical MALDI UV lasers, so direct UV matrix ionization cannot be considered the common mechanism for primary ions generation.

On the other hand, thermal energy released with the MALDI plume can make up the difference between photon energy and ionization potentials, at least for some matrices.
Energy Pooling and Multicenter Models

According to this mechanism, two or more separately excited matrix molecules pool their energy to yield one matrix radical cation or a highly excited matrix molecule:

\[ M \xrightarrow{h\nu} M^* \quad M \xrightarrow{h\nu} M^* \rightarrow M + M^{**} \xrightarrow{h\nu} M^{+\cdot} + e^- \]

Matrix molecules clusters could be also involved in this process, due to their usually lower IPs:

\[ 2h\nu \quad MM \rightarrow M^*M^* \rightarrow M + M^{+\cdot} + e^- \text{ (or other matrix ions)} \]

\[ M^*M^* + A \rightarrow MM + A^{+\cdot} + e^- \text{ (or other analyte ions)}. \]
For statistical reasons, at typical MALDI laser fluences it is more likely that a neighbor of an excited matrix molecule will be the next to become excited, rather than the excited molecule will be hit by a second photon.

An energy pooling mechanism for ion formation will, therefore, be statistically favored, if it can occur.

Time delayed two-pulse experiments show that, on a time scale of about 10 ns, MALDI depends on the number of photons delivered, not on the rate (irradiance W/cm$^2$) at which they arrive.

The energy pooling mechanism is attractive because it explains how energetic processes can occur in a diffusely excited solid.

It is also plausible, because:

- strong interactions between closely packed chromophores in solids are common
- clusters are known to be produced in MALDI plumes.
A schematic diagram of photoionization and subsequent reaction pathways in MALDI MS was proposed by Ehring, Karas, and Hillenkamp in 1992:

[Diagram showing energy pooling, photo-excitation, photo-ionizations, photochemical reactions, and observed ions]
Pooling ionization mechanisms include at least three states of the matrix: the ground electronic ($S_0$) and two excited electronic states, $S_1$ and $S_n$.

In the case of a single matrix molecule, the following scheme can be hypothesised:

The first excited state ($S_1$) has been well-characterized by spectroscopy in the case of DHB and lies at 3.4662 eV in the gas phase. Here, it is considered to have the same energy as a single UV laser photon.

The higher state ($S_n$) is simply higher than the $S_1$ by one laser photon energy but it is below the ion state.

Typical UV-MALDI laser wavelengths are 355 nm (3.49 eV, tripled Nd:YAG laser) or 337 nm (3.68 eV, $N_2$ laser), so two photon states are indeed below the ionization potential, for example, of DHB at 8.054 eV.
If bimolecular processes are considered, the following schemes can be supposed:

In pooling mechanisms, de-excitation from $S_1$ to $S_0$ (which can be radiative or non-radiative) provides energy either for excitation of another electron from $S_1$ to $S_n$, or for ionization of an electron in a $S_n$ level.

Energy pooling can occur only if the electronic states of the two excited molecules can interact. In the case of typical MALDI matrices, this occurs through interactions between aromatic $\pi$-electron systems of the stacked, ordered matrix molecules in the solid.
Inter-molecular hopping is the process enabling transformation of neighbouring matrix molecules in excited species, for example $S_1$-$S_1$, starting from distant excited matrix molecules:

Energy pooling can then occur between neighbouring excited molecules:
An experimental demonstration of $S_1 - S_1$ energy pooling is provided by reporting the matrix fluorescence efficiency as a function of laser pulse energy (fluence), as shown for the 2,5-DHB matrix:

Fluorescence efficiency is significantly decreased at the increase of the number of laser photons hitting the matrix, since the probability of generation of neighbouring excited molecules, and then of energy pooling, competitive with fluorescence, is increased.
Theoretical calculations can be used to predict the populations of excited states and the primary ions distributions in the first stage of MALDI plume generation.

The following plot is referred to the first layer of a DHB matrix hit by a 5 ns laser pulse at 355 nm.
Since primary ionization involves multiple excitation, hopping and pooling steps, it is dependent on the laser fluence in a highly nonlinear manner. In particular, a fluence threshold for ionization can be individuated for a specific laser on a certain matrix.

In the figure the dependence of MALDI efficiency on fluence is reported for N\(_2\) and Nd:YAG lasers on a DHB matrix:

Upper curves represent yields from the top layer, lower curves the integrated yields for all the emitted material.

Note the relatively slight variation of efficiency when the Nd:YAG laser pulse width is changed.
Increased acidity due to electronic excitation makes the probability of a proton transfer between an excited matrix molecule and an analyte or ground-state molecule nearby higher:

\[
M + h\nu \rightarrow M^* \\
M^* + A \rightarrow (M - H)^- + AH^+ \\
M^* + M \rightarrow (M - H)^- + MH^+
\]

ESPT could possibly occur after an intramolecular proton transfer, from hydroxyl to carbonyl oxygen, in ortho-hydroxy-carboxylic acids, common among MALDI matrices:

The generated ortho-hydroxy carbonyl structure has been proposed to improve ion yields by proton transfer to the analyte.
Disproportionation Reactions

After photoexcitation of a strongly coupled matrix pair (concerted reaction), disproportionation to anion/cation couples can occur:

\[ 2M \xrightarrow{nh\nu} (MM)^* \rightarrow (M - H)^- + (M + H)^+ \]

\[ 2M \xrightarrow{nh\nu} (MM)^* \rightarrow M^- + M^+ \]

The process is accessible by 2 photons, meeting typical energy requirements (in the case of DHB 13.8 eV would be required to eject an electron):

<table>
<thead>
<tr>
<th>Matrix</th>
<th>kcal/mol</th>
<th>eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHB</td>
<td>121</td>
<td>5.24</td>
</tr>
<tr>
<td>3HPA</td>
<td>120</td>
<td>5.18</td>
</tr>
<tr>
<td>NA</td>
<td>118</td>
<td>5.10</td>
</tr>
<tr>
<td>THA</td>
<td>113</td>
<td>4.89</td>
</tr>
</tbody>
</table>

DHB = 2,5 dihydroxybenzoic acid, 3HPA = 3-hydroxypicolinic acid, NA = nicotinic acid, THA = 2,4,6-trihydroxyacetophenone
Desorption of Preformed Ions

Preformed ions are merely released by the laser pulse. This mechanism is potentially most relevant for MALDI-MS of compounds that are intrinsically ionic in character:

- ionized compounds
- metal – ion complexes, such as those formed by crown ethers, ionophores or metal-binding proteins
- pre-protonated compounds

Note that:

- ions generated into the gas phase cannot be distinguished from preformed ones;
- negative preformed ions are not common.
Thermal ionization

This process is negligible in normal UV MALDI as the temperature of desorbed molecules is about 500 K.

An increase in its probability occurs in LDI (e.g. laser desorption ionization without matrix), where temperatures around 3000 K are measured.

Thermal ionization could have some role in two-phase MALDI (i.e. when solid particles are embedded in the matrix).

If ionization occurs directly at the particle surface and under thermal equilibrium conditions, the Saha-Langmuir equation describes the ratio between the numbers of ions ($Y_1$) and neutrals ($Y_0$) for a species $i$:

$$\frac{Y_1}{Y_0} = \frac{g_1}{g_0} \exp \left( \frac{\phi - P_i}{kT} \right)$$

Where:

- $g_1/g_0$ is the ratio of statistical weights for ions and neutrals,
- $\phi$ is the work function of the particle surface
- $P_i$ is the ionization potential for species $i$. 
For a typical $\phi - P_i$ difference (about -5 eV, related to the graphite-glycerol couple) and a 1000 K temperature a negligible ionization extent would be obtained, which is in contrast with the good efficiency of two-phase MALDI.

On the other hand, if nanometric particles are present (diameters of a few tens of nm), the local temperature on each particle can reach 10000 K, thus providing a $Y_1/Y_0$ value close to $10^{-3}$, comparable to that of UV MALDI.

Improvements can be obtained also by raising the $\phi$ value, e.g. through oxidation of the particle surface or use of special high work function materials like nitrides (e.g. TiN).

Thermal ionization can also occur, at least in principle, in the matrix bulk.

The process is actually a disproportionation and electron affinities (EA) of the matrix have to be included in the Saha-Langmuir equation:

$$\Delta H \\ 2M \rightarrow M^- + M^+$$

$$\frac{Y_1}{Y_0} = \frac{g_1}{g_0} \exp \left( \frac{EA - P_i}{kT} \right)$$

As EA values (about 1 eV) are quite lower than $\phi$ ones, very high temperatures are required for bulk thermal ionization in MALDI.
Proposed Secondary Ionization Mechanisms

In MALDI the term secondary ions is referred to ionized species generated during matrix-matrix or matrix-analyte reactions subsequent to the formation of the MALDI plume.

The main mechanisms hypothesized for secondary ion generation are:

✓ Gas-Phase Proton Transfer / Electron Capture
✓ Gas-Phase Cationization
✓ Electron Transfer
✓ Charge Compensation
Gas-Phase Proton Transfer/Electron Capture

Matrix-matrix reactions

Within the MALDI plume primary matrix ions can interact with matrix neutral molecules through a proton transfer:

\[ M^+ + M \rightarrow MH^+ + (M-H)^\cdot \]

The resulting radical \((M-H)^\cdot\) may capture a free electron to form the even-electron anion \((M-H)^-\):

\[ (M-H)^\cdot + e^- \rightarrow (M-H)^- \]

The same species can be generated also by a dissociative electron capture reaction, likely interesting a OH group on the matrix molecule:

\[ M + e^- \rightarrow (M - H)^- + H^\cdot \]
Matrix ion – neutral analyte reactions

Protonated matrix molecules can transfer a $\text{H}^+$ to an analyte molecule:

$$ (M + H)^+ + A \rightarrow M + (A + H)^+ $$

Proton affinities of peptides and proteins, typical analytes for MALDI-MS, are in the order of 240 kcal/mol, whereas those of MALDI matrices vary between 183 and 225 kcal/mol.

Therefore, proton transfer to a neutral gas-phase peptide or protein will be thermodynamically favorable almost in all cases.

Sensitivity will also be improved if the protein or peptide contains one or more basic amino acids (lysine, arginine, etc.).

For matrices with a low PA, and/or analytes with a high PA, the proton-transfer reaction will be quite exothermic. This excess energy will be deposited into the reaction partners as internal excitation and can lead to increased analyte fragmentation.
De-protonated matrix molecules could potentially capture a H$^+$ from an analyte molecule:

\[
(M - H)^- + A \rightarrow M + (A - H)^-
\]

However, due to the generally low gas phase basicity of matrix anions, compared to typical analytes, this process appears to be quite unlikely.

A dissociative electron capture by the analyte molecule (similar to that occurring on matrix molecules) could eventually lead to a deprotonated analyte molecule:

\[
A + e^- \rightarrow (A - H)^- + H^*
\]
Gas-Phase Cationization

Cationization is actually a cation transfer to the analyte molecule, similar to proton transfer.

Such a process can occur with:

- $K^+$ or $Na^+$ contaminants
- cations purposely added to the matrix
- laser irradiation of neat salts

It can be enhanced by delayed extraction, i.e. by a delay in the application of electrical fields required to transfer ions to the mass analyser.

Cationization is hindered by the use of complexing matrices.
Electron Transfer Reactions

In this process an electron is transferred to a matrix radical cation from an analyte molecule, provided the ionization potential of the latter is lower:

\[ M^{+\bullet} + A \rightarrow M + A^{+\bullet} \]

Conjugated oligomers, like terthiyophene, can be used as electron transfer matrices in the MALDI-MS analysis of special compounds like ferrocenes:
The choice of the matrix can have a great influence on the MALDI-MS response for a specific analyte.

In the following example, a signal is observed for triphenyl-phosphine only when 1,4-diphenyl-1,3-butadiene is used as a matrix, since the latter has a higher IP. No signal is observed when perylene, having a lower IP than the analyte, is used:
Charge compensation through charge ejection

In many cases ions with surprising compositions, but still having a single charge (positive or negative), are found in MALDI-MS spectra.

As an example, ions corresponding to adducts between DHB and Ca$^{2+}$ ions, with formula $[\text{DHB}_n\text{Ca}_{n-1}]^+$, have been observed.

The net single negative charge, i.e. the partial compensation of the Ca$^{2+}$ ions, arises from ejection of two H$^+$ ions from DHB molecules for each Ca$^{2+}$ ion incorporated.

The phenomenon of charge compensation via charge ejection has been observed also for adducts involving the analyte molecules, both positively and negatively charged (e.g. adducts like $[\text{A}+2\text{Na}+\text{K}-4\text{H}]^-$ have been observed for certain peptides).

In some cases, even the reduction (by electrons) of metal cations involved in adducts (e.g. Cu$^{2+}$ to Cu$^+$) can lead to charge compensation.
Incidence of primary and secondary ionization mechanisms in different forms of MALDI

<table>
<thead>
<tr>
<th></th>
<th>UV MALDI</th>
<th>IR MALDI</th>
<th>2-Phase MALDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P+</td>
<td>NP+</td>
<td>P−</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPI</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Pooling</td>
<td>●●</td>
<td>●●</td>
<td>●●</td>
</tr>
<tr>
<td>ESPT</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Disproportionation</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Preformed</td>
<td>●●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Thermal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H⁺ Transfer</td>
<td>●●</td>
<td>●●</td>
<td>●●</td>
</tr>
<tr>
<td>e⁻ Capture &amp; H⁺ Transfer</td>
<td>●●</td>
<td>●●</td>
<td>●●</td>
</tr>
<tr>
<td>Cationization</td>
<td>●●</td>
<td>●●</td>
<td>●●</td>
</tr>
<tr>
<td>e⁻ Transfer</td>
<td>●</td>
<td>●</td>
<td>●●</td>
</tr>
<tr>
<td>Ejection</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P/NP: polar/non polar analytes; ●● highly active; ● active; ? sometimes active; ?? Possibly active but not well studied.

Due to relative lack of experimental data, the UV NP- and both IR negative categories are not included in the scheme.
**Hypothesised origins of ions observed in MALDI spectra for different analytes**

<table>
<thead>
<tr>
<th>Category</th>
<th>Desorption of pre-formed ions</th>
<th>Gas-phase proton transfer</th>
<th>Gas-phase cationization, anion addition</th>
<th>Electron transfer</th>
<th>Charge ejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptides, proteins</td>
<td>tagged: $A^+$</td>
<td>$A^+ (A + H)^+$</td>
<td>$(A + C)^+$</td>
<td>$(A + C(II) - H)^+$</td>
<td></td>
</tr>
<tr>
<td>Metalloproteins</td>
<td>$(A + C)^+$</td>
<td>$(A + H)^+$</td>
<td>$(A + C)^+$</td>
<td>$(A + C(II) - H)^+$</td>
<td></td>
</tr>
<tr>
<td>Ionophores, Metal complex ligands</td>
<td>$(A + C)^+$</td>
<td>$(A + H)^+$</td>
<td>$(A + C)^+$</td>
<td>$(A + C(II) - H)^+$</td>
<td></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>$(A + H)^+$</td>
<td>$(A + Na/K)^+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>$(A - H)^-$</td>
<td>$(A - H)^-$</td>
<td>$(A + C)^+$</td>
<td>$(A + C - nH)^-$</td>
<td></td>
</tr>
<tr>
<td>Polar Polymers</td>
<td>$(A + H)^+$</td>
<td>$(A + Na)^+$</td>
<td>$(A + C)^+$</td>
<td>$(A + C(II) - H)^+$</td>
<td></td>
</tr>
<tr>
<td>Apolar Polymers</td>
<td>$(A - H)^-$</td>
<td>$(A + Ag)^+$</td>
<td></td>
<td>$(A + C(II) - H)^+$</td>
<td></td>
</tr>
<tr>
<td>Fullerenes, Fullerene Derivatives</td>
<td>$(A + H)^+$</td>
<td>$(A + C)^+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-IP compounds (e.g. Ferrocenes, metalloycles)</td>
<td>$(A - H)^-$</td>
<td>$(A - H)^-$</td>
<td>$(A + C)^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly acidic analytes (e.g. sulfonated dyes)</td>
<td>$(A - H)^-$</td>
<td>$(A - H)^-$</td>
<td>$(A + X)^-$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MALDI signal intensities and suppression effects

Even when homogeneous samples are prepared, the concentration dependence of the MALDI signal for an analyte can be dramatically non-linear.

In this example the ratio of MALDI signals between the matrix 3-amino-quinoline (3AQ) and the analyte 3-morpholine-propan-sulphonic acid (MOPS) has a clearly non-linear dependence on their molar ratio:
The more pronounced decrease of the matrix signal, compared to the analyte one, when their molar ratio is decreased, can be interpreted with a limiting reagent effect occurring during processes leading to secondary ions.

If analyte molecules are relatively abundant in the MALDI plume, matrix primary ions become limiting reagents and are almost completely turned into neutral species, to ionize analyte neutrals.

In extreme cases, the matrix signals can be completely suppressed:
A combination of matrix (M) and analyte (A) suppression effects can be observed in MALDI spectra when the analyte is actually a mixture of compounds:

In this example compound A is preferentially ionized and components B-E of the mixture are almost completely suppressed (with matrix) at the lowest M/A ratio.
It is worth noting that, when observed, suppression involves all types of ions of a matrix or analyte, e.g. both radical cations and protonated or cationized ions.

In this example, the protonated ion for Substance P is able to suppress the [M+ Na]⁺ (and also the [M+K]⁺) ion of valinomycin.

This phenomenon suggests that a complete inter-convertion occurs in the MALDI plume between different ions related to the same species:

\[
\begin{align*}
P + MH^+ &\rightarrow PH^+ + M \\
M + MNa^+ &\rightarrow MH^+ + [M - H]Na \\
ValNa^+ + M &\rightarrow MNa^+ + Val \\
P + ValNa^+ + M &\rightarrow PH^+ + Val + [M - H]Na
\end{align*}
\]
## Advantages and disadvantages of MALDI-MS

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Practical mass range of up to 300 000 Da. Species of much greater mass have been observed using a high current detector</td>
<td>Matrix background interferes in spectra of analytes having MW &lt; 700 Da. This background interference is highly dependent on the matrix material</td>
</tr>
<tr>
<td>Typical sensitivity in the order of low femtomole to low picomole; attomole sensitivity is possible</td>
<td>Possibility of photodegradation by laser desorption/ionization</td>
</tr>
<tr>
<td>Soft ionization with little to no fragmentation observed</td>
<td>Acidic matrix used in MALDI may cause chemical degradation on some compounds</td>
</tr>
<tr>
<td>Tolerance of salts in millimolar concentrations</td>
<td>Quantification is very difficult</td>
</tr>
<tr>
<td>Suitable for the analysis of complex mixtures</td>
<td></td>
</tr>
</tbody>
</table>
MALDI-MS spectrum for Bovine Insulin in Sinapinic Acid (SA)

- \([M+H]^+\)
- \([M+2H]^{2+}\)
- \([M+Matrix+H]^+\)
- \([2M+H]^+\)
- \([3M+H]^+\)
MALDI-MS spectrum for a mixture of 3 Proteins in SA

- Bovine Insulin, MW 5733.5
- Cytochrome C, MW 12360.1
- Trypsinogen, MW 23981

Counts

m/z

Ins$^+$

Cyt.C$^+$

Ins$^{2+}$

Cyt.C$^{2+}$

Try$^{2+}$

2 Cyt.C$^+$

Try$^+$
DIOS is a relatively recent (1999) matrix-less desorption/ionization strategy for biomolecular mass spectrometry, based on pulsed laser desorption from a porous silicon surface. The main advantage of DIOS over MALDI is the reduced number of interferent peaks (due to matrix absence).
DIOS substrate preparation

Effective porous silicon samples for DIOS can be prepared from either n- or p-type silicon, using a specific etching.

For n-type:

P-doped, (100) orientation, 0.65 Ω cm resistivity Si wafers

Etching for 1–3 min at +71 mA cm⁻² current density with illumination by a 300-W tungsten filament bulb in a 1:1 solution of ethanol/49% HF (aq).

For p-type:

B-doped, (100) orientation, 0.01 Ω cm resistivity Si wafers

Etching for 3 h at 37 mA cm⁻² current density in the dark in a 1:1 solution of ethanol/49% HF (aq).
The etching procedure makes the silicon surface porous and thus able to trap the analyte molecules. Because of Si high absorptivity in the ultraviolet, the substrate acts as an energy receptacle for the laser radiation.

A SEM image shows how the silicon surface looks like, after preparation for DIOS-MS acquisitions:
Influence of experimental parameters on sensitivity

Pore size - Both n-type mesoporous samples and p-type micro- or mesoporous samples are effective in generating signals, but the surfaces with smaller pore sizes typically give a more intense ion signal.

Surface modification - hydrogen (native), dodecyl, ethyl-phenyl (-CH$_2$CH$_2$C$_6$H$_5$) and oxide surface modifications have been tested; the more hydrophobic surfaces, and in particular the ethyl-phenyl-terminated surface, typically give better signals for the same quantity of analyte from an aqueous medium.

Sample deposition - Dissolving the sample in methanol/H$_2$O at a 1:1 v/v ratio also provides a stronger signal, indicating that analyte penetration into porous silicon is important.
Mechanisms for DIOS

The energy for analyte release from the surface may be transferred from silicon to the trapped analyte through vibrational pathways or as a result of the rapid heating of porous silicon producing $\text{H}_2$ that releases the analyte.

Alternatively, as porous silicon absorbs hydrocarbons from air or while under vacuum, rapid heating/vaporization of trapped hydrocarbon contaminants or solvent molecules may increase the vaporization and ionization of the analyte embedded in the porous silicon.

The observation of DIOS MS background ions with $m/z < 100$, consistent with aliphatic hydrocarbons, at high laser intensities indicates that they may play a role in desorption/ionization.
Reduction of interferent peaks in DIOS spectra

A comparison with a MALDI MS spectrum of the common matrix α-cyano-4-hydroxy cinnamic acid emphasises the number of matrix peaks present in the same m/z range, that makes MALDI-MS analysis of small analytes quite difficult.
In this application, even Na\(^+\) ions (m/z 23), responsible for the generation of a [M+Na]\(^+\) ion, at m/z 315, can be easily detected.
Surface modifications of DIOS substrates

The reactivity of silanol groups introduced by etching at the silicon surface can be exploited for several surface modifications of DIOS substrates:

Pendant pentafluoro-phenyl groups make the surface hydrophobic, thus enhancing the MS signal for non-polar drugs.
midazolam propafenone verapamil

midazolam propafenone verapamil
The perfluorophenyl silylated modified chip has been applied also to DIOS-MS of extremely low amounts of peptides:

Zepto = $10^{-21}$
Yocto = $10^{-24}$
Pendant propyl-amino groups make the surface hydrophilic, thus increasing the MS signal for polar analytes like carbohydrates.