ElectroSpray Ionization (ESI)
A key figure in the early history of electrospray: Jean Antoine Nollet

A key role in the first experiments along the way to Electrospray Ionization was played in the 18th century by Jean-Antoine Nollet, a French clergyman but also experimental physicist, leading member of the Paris Academy of Science.

He constructed one of the first electrometers and developed a theory of electrical attraction and repulsion that supposed the existence of a continuous flow of electrical matter between charged bodies.

Nollet was the first professor of experimental physics at the University of Paris.
In order to test his theory that electricity traveled far and fast Jean-Antoine Nollet set up a famous experiment.

On a spring day in 1746 he sent 200 of his monks out in a line 1 mile long. Between each pair of monks was a 25-foot iron wire. Once the monks were properly aligned, Nollet hooked up a Leyden jar (a simple capacitor consisting of a glass bottle with conducting metal foil coating the inner and outer surfaces) to the end of the line.

As a result, all the monks started swearing, contorting, or otherwise reacting simultaneously to the shock, confirming the conduction of electrical charge through their bodies.

In a further demonstration, Nollet discharged a Leyden jar in front of King Louis XV at Versailles by sending current through a chain of 180 Royal Guards.
1750: the first electrostatic spraying experiment

Nollet observed that when a person was connected to a high-voltage generator he/she did not bleed normally after being cut. Actually blood was sprayed through the cut!

In a less macabre version of the experiment, Nollet showed the astonishing efficiency of electrostatic spraying.

As shown in the picture, application, through an insulated chain, of a high d.c. voltage generated by a rotating glass ball to various conducting devices containing a liquid and having an orifice, produced an evident spray.
Further milestones in the ElectroSpray history

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
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<tbody>
<tr>
<td>1917</td>
<td>First modern report on the electrospray process by J. Zeleny (<em>Phys. Rev.</em>, 10, 1-6). The paper described how a high electrical potential applied to a capillary caused the solvent to break into fine threads disintegrating to small droplets.</td>
</tr>
<tr>
<td>1968</td>
<td>Dole et al., introduced electrospray process as a method of generating gas phase ions in an attempt to determine the mass of polystyrene macromolecules by ion retardation and ion mobility measurements (M. Dole et al., <em>J. Chem. Phys.</em>, 49, 2240)</td>
</tr>
<tr>
<td>1984</td>
<td>The first experimental description of electrospray ionisation mass spectrometry (ESI – MS) was provided (Yamashita and Fenn <em>J. Phys. Chem.</em>, 88, 4451)</td>
</tr>
<tr>
<td>1988</td>
<td>The potential of ESI – MS for the analysis of large biomolecules was recognised for the first time by Meng et al., (<em>Z. Phys. D</em> 10, 361)</td>
</tr>
<tr>
<td>2002</td>
<td>J.B. Fenn - Nobel Prize in Chemistry 2002 &quot;for the development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules&quot;</td>
</tr>
</tbody>
</table>
ESI: an example of atmospheric pressure ion (API) source
Longitudinal configuration

Orthogonal configuration
The electrospray process: a phenomenological view

1. Entrance to mass spec
2. Liquid
3. Charge redistribution
4. Taylor-cone formation
5. Formation of charged droplets and ions
Nebulization (pneumatic assistance) in the ESI process

The assistance by a inert gas, flowing co-axially or normally to the electrosprayed solution, improves desolvation of droplets, increasing the number of generated ions.

Conventional unassisted ESI works best at flow rates of 1-20 μL/min. Pneumatic assistance enables working with flow rates up to 10 fold higher.
The ESI mechanism: basic steps

The ESI process proceeds essentially through three steps:

1. Production of charged droplets at the ESI capillary tip;

2. Shrinkage of charged droplets by solvent evaporation at constant charge; when the radius decreases below the Rayleigh value, fission into smaller droplets occurs;

3. Production of gas-phase ions
Production of charged droplets at the ESI capillary tip

While pumped through the spray needle kept at high voltage the ESI solution forms the so-called Taylor cone, resulting from mutual repulsion between ions with the same charge, accumulated in solution at the needle tip.
The electric field at the capillary tip, \( E \), is given by:

\[ E = \frac{2V}{r \cdot \ln \left( \frac{4d}{r} \right)} \]

The Taylor cone results from a balance between electrostatic force and surface tension:

For \( V = 2 \text{ kV}, r = 0.2 \text{ mm} \) and \( d = 2 \text{ cm} \), an electric field of \( 3 \times 10^6 \text{ V m}^{-1} \) can be calculated.
The very high electric field applied at the electrospray (metal) capillary tip leads to a partial charge separation because the field penetrates partly past the surface of the liquid at the tip of the capillary.

In the positive ion mode, positive ions are enriched at the surface of the liquid located at the capillary tip whereas negative ions are driven towards the inside of the capillary.

The repulsion of the positive ions at the surface and the pull of the electric field on the positive ions overcome the surface tension of the liquid and make it expand into the Taylor cone, the tip of which, being the least stable point, elongates into a liquid filament. This filament breaks up into individual charged droplets.
The onset of the electrospray requires an electric field or an electric potential given by:

\[ E_0 = \left( \frac{2 \gamma \cos 49^\circ}{\varepsilon_0 r_c} \right)^{1/2} \]

\[ V \approx \sqrt{\frac{r_c \gamma \cos 49^\circ}{2 \varepsilon_0}} \cdot \ln \frac{4d}{r_c} \]

where:

\( \gamma \) is the surface tension of the liquid

\( 49^\circ \) is the half-angle of the Taylor cone

\( \varepsilon_0 \) is the permittivity of vacuum

\( r_c \) is the capillary radius

\( d \) is the distance between needle tip and metal plate
The value of the onset potential can be found by plotting the current obtained at the ESI interface and the MS signal, related to generated ions, versus the tip potential:
If high potentials are applied, a corona discharge can be produced between needle tip and metal plate: the current is increased but charge recombination leads to a MS signal decrease.
Influence of solvent surface tension on the ESI onset voltage

Solvent surface tension has a significant influence on the onset potential of the ElectroSpray Ionization:

\[ V \approx \sqrt{\frac{\gamma \cos 49}{2 \varepsilon_0}} \cdot \ln \frac{4d}{r} \]

Onset potentials calculated with:
\[ \varepsilon_0 = 8.85 \times 10^{-12} \, \text{C}^2\text{J}^{-1}\text{m}^{-1}, \quad r = 0.1 \, \text{mm}, \quad d = 40 \, \text{mm} \]

<table>
<thead>
<tr>
<th></th>
<th>CH₃OH</th>
<th>CH₃CN</th>
<th>(CH₃)₂SO</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{onset}} ) (kV)</td>
<td>2.2</td>
<td>2.5</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>( \gamma ) (N/m²)</td>
<td>0.0226</td>
<td>0.030</td>
<td>0.043</td>
<td>0.073</td>
</tr>
</tbody>
</table>
Requirements on solvent (mobile phase) for optimal ESI

Surface tension is just one of the solvent properties that need to be evaluated carefully for an optimal ESI.

Low viscosity is also needed, to facilitate desolvation from droplets.

High values of the solvation $\Delta H$ are required, to make final ion desorption easier.

Further requisites concern electrostatic interactions and acid/base reactions. Thus a good ESI solvent needs to have:

- a high dielectric constant (relative permittivity)
- a high polarity
Comparison between properties of typical HPLC (reverse or normal phase) solvents in relation to ESI sustainment:

<table>
<thead>
<tr>
<th></th>
<th>Surface Tension (dyn/cm)</th>
<th>Dipole (D)</th>
<th>Dielectric Constant (@20°C)</th>
<th>Viscosity (cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>22.55</td>
<td>2.87</td>
<td>32.7</td>
<td>0.59</td>
</tr>
<tr>
<td>Ethanol</td>
<td>22.32</td>
<td>1.66</td>
<td>24.55</td>
<td>1.10</td>
</tr>
<tr>
<td>Propanol</td>
<td>23.70</td>
<td>3.09</td>
<td>20.33</td>
<td>2.30</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>21.79</td>
<td>1.69</td>
<td>19.92</td>
<td>2.40</td>
</tr>
<tr>
<td>Butanol</td>
<td>22.98</td>
<td>1.75</td>
<td>17.51</td>
<td>2.98</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>19.10</td>
<td>3.44</td>
<td>37.50</td>
<td>0.38</td>
</tr>
<tr>
<td>Water</td>
<td>72.80</td>
<td>1.87</td>
<td>80.10</td>
<td>1.00</td>
</tr>
<tr>
<td>DMF (1)</td>
<td>36.76</td>
<td>3.86</td>
<td>36.71</td>
<td>0.92</td>
</tr>
<tr>
<td>DMSO (1)</td>
<td>13.63</td>
<td>4.10</td>
<td>46.68</td>
<td>2.24</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>23.22</td>
<td>2.69</td>
<td>20.70</td>
<td>0.93</td>
</tr>
<tr>
<td>Acetone</td>
<td>28.53</td>
<td>0.31</td>
<td>2.38</td>
<td>0.59</td>
</tr>
<tr>
<td>Toluene</td>
<td>17.91</td>
<td>0.08</td>
<td>1.88</td>
<td>0.31</td>
</tr>
<tr>
<td>Hexane</td>
<td>28.12</td>
<td>1.15</td>
<td>4.81</td>
<td>0.57</td>
</tr>
<tr>
<td>Chloroform</td>
<td>24.98</td>
<td>0.0</td>
<td>2.02</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Water has good polarity and dielectric constant and a fair viscosity, yet its surface tension is very high.

Water-methanol or water-acetonitrile mixtures represent good compromises between the three parameters, thus RPC and HILIC can be easily interfaced to ESI-MS, whereas NPC can pose significant problems.
If a gradient elution is adopted for RP-LC-ESI-MS analyses one should expect an increase in sensitivity when the percentage of methanol or acetonitrile is increased.

This behavior can be confirmed experimentally by comparing the ESI-MS responses of homologous compounds at the same concentration and regularly eluting along the chromatographic trace, like linear aliphatic alcohols with increasing side chains:
Effect of solution (LC mobile phase) pH

In the case of acid or basic analytes the distribution between neutral and charged forms in solution is deeply influenced by the pH, with remarkable effects on the ESI-MS sensitivities.
In the case of amphoteric analytes, like aminoacids, a different ion mode can be adopted according to the dominating charge, i.e. to the solution pH:

A pH interval will exist in which the zwitterionic form will prevail over the neutral or charged ones: a decrease in the ESI-MS response will be then observed, apart from the ion mode adopted.
Shrinkage of charged droplets by solvent evaporation

Big charged droplets are generated initially at the tip of the liquid filament protruding from the Taylor cone:

The initial droplet radius and charge are given by the following equations:

\[ R \approx \left( \frac{V_f \varepsilon}{K} \right)^{1/3} \]

\[ q \approx 0.7 \left[ 8\pi \gamma R^3 \right]^{1/2} \]

where:

\( V_f \), \( \varepsilon \) and \( K \) are the solution flow rate, permittivity and conductivity, respectively.
The parent droplet is actually split into a single droplet, having a slightly lower radius, and a population of about 20 small droplets, carrying off 2% of the mass and 15% of the charge:

\[ q = 8\pi (\epsilon_0 \gamma R^3)^{1/2} \]

When shrinking starts, the droplet radius is reduced while the charge is still the same: the mutual charge repulsion overcomes the solvent surface tension when the so-called Rayleigh limit is reached:

The parent droplet is actually split into a single droplet, having a slightly lower radius, and a population of about 20 small droplets, carrying off 2% of the mass and 15% of the charge:

\[ N = \text{number of elementary charges} \]
\[ R = \text{droplet radius in } \mu\text{m}. \]
The first fission triggers a fission cascade: further shrinking of the bigger droplet arising from a fission leads to reach the Rayleigh limit in a $\Delta t$ time and then to a new fission, according to a model developed by Kebarle and Tang in 1993.
Production of gas-phase ions: Charge Residue Model (CRM)

The combination of solvent evaporation and Coulombic fission, proposed, in a qualitative way, by Dole et alt. already in 1968, produces ultimate nanometric droplets, thus containing, reasonably, only one molecule of solute.

According to the CRM this molecule becomes a free gas-phase ion by retaining the droplet charge until the solvent evaporates.

Since the offspring droplets (i.e. those generated by subsequent fissions) multiply very rapidly, by a factor of 20 at every fission of the parent droplet, ion signal intensity due to single ions $M^+$ may dominate the spectra when the initial concentration $C_{MX}$ is not too high, i.e. within the conventional ESI-MS concentration range (up to $10^{-3}$ M).

The relevance of more complex ions like $[M_x(MX)_n]^{x+}$ (with $X$ = counterion and $x$ and $n$ assuming low integer values) in ESI-MS spectra is higher the higher the analyte concentration.
Production of gas-phase ions: Ion Evaporation Model (IEM)

The IEM was elaborated by Iribarne and Thomson between 1976 and 1979 and can be considered complementary to the CRM.

According to IEM, when solvent evaporation and Coulomb droplet fissions have reduced the size of the charged droplets to a certain radius $R$ ($R \approx 10 \text{ nm}$, $N \approx 100$), the electric field on their surface becomes strong enough to overcome solvatation forces and lift a solute ion from the droplet surface into ambient gas:
The main differences between IEM and CRM are:

✓ ion evaporation is on the $\mu$s time scale and thus very fast;

✓ the evaluated charge, $q_i$, required for ion evaporation is lower than the charge required for Coulomb fission. This means that, once possible, ion evaporation would keep the droplet charge low, preventing further Coulombic fission;

✓ the proportion of gas phase ions $M^+$ relative to that of complex $[(M^+)_x(MX)_n]^{x+}$ ions might be higher than that expected with the CRM.

Ion species observed in a ESI-MS spectrum are reasonably formed by both IEM and CRM.

However, small ions such as $M^+$ are likely produced by IEM, whereas larger ions like $(M^+)_x(MX)_n$, where $x > 1$ or 2 or 3 and $n >> 1$, are more likely produced by CRM.
IEM: rate of ion evaporation from the droplets

The ion evaporation rate constant can be expressed by the Iribarne equation:

\[ k_I = \frac{k_B T}{h} e^{-\Delta G^\ddagger/RT} \]

where \( k_B \) is the Boltzmann constant and \( \Delta G^\ddagger \) is the energy barrier between the initial state and the transition state:

- **initial state**
  - \( R = 8 \text{ nm} \)
  - \( N = 70 \)

- **transition state**
  - \( R = 8 \text{ nm} \)
  - \( N = 69 \)
In this scheme the transition state occurs after the ion has left the droplet.

The maximum of the energy barrier is expected when the evaporating ion is at a 0.5 nm distance from the droplet surface.

According to the IEM, $\Delta G^\ddagger$ consists of two contributions:

$$\Delta G^\ddagger = -\Delta G^\circ_{\text{sol}} + \Delta G^\ddagger(E)$$

$-\Delta G^\circ_{\text{sol}}$ is the free energy related to ion desolvation

$\Delta G^\ddagger(E)$ is a function of the electrical field perpendicular to the charged droplet surface, which can be derived from Coulomb’s law, due to the spherically symmetric distribution of charges ($q = Ne$) on the droplet:

$$E = \frac{q}{4\pi \varepsilon_0 R^2}$$
CRM versus IEM

Small ions

IEM provides much better fit of MS spectral data than CRM does. Assuming that an IEM-like process occurs at the very last stages of CRM, where the gas phase ions are produced, the distinction between IEM and CRM is very subtle: the first model would predict ion evaporation already from 10 nm–sized droplets, the second would actually invoke the same process but from smaller droplets (3 nm).

Macro-ions

Multi-protonated globular proteins (i.e. not or partly denatured in the solution used for ESI) are most probably produced by CRM. Open (unfolded) multi-charged structures like denaturated proteins could be produced by either CRM or IEM.
Dependence of ESI ion types on analyte properties

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Positive Ions</th>
<th>Negative ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Non-polar</td>
<td>[M+H]^+ , [M+alkali]^+</td>
<td>[M-H], [M+A]^−</td>
</tr>
<tr>
<td>Medium to high polarity;</td>
<td>[M+H_n]^n+ , [M+alkali_n]^n+</td>
<td>[M-H_n]^n− , [M-A_n]^n−</td>
</tr>
<tr>
<td>Number of charges generally increases with size and basicity/ acidity</td>
<td>Exchange [M-H_n+alkali_{n+x}]^{x+}</td>
<td>Exchange M-Hn+ alkali_{n-x}^{x−}</td>
</tr>
<tr>
<td></td>
<td>Clusters [M_m+H_n]^n+ , [M_m+alkali_n]^n+</td>
<td>Clusters [M_m-H_n]^n− ,</td>
</tr>
<tr>
<td>Ionic (C^{+}A^{−})</td>
<td>C^{+} , [C_n+A_{n−1}]^{+}</td>
<td>A^{−} , [C_{n−1}+A_n]^{−}</td>
</tr>
</tbody>
</table>

Note that only even-electron ions are usually expected in ESI-MS and molecular ions (like M^{+} obtained in EI-MS) are observed only in special cases.
In particular, radical (odd electron) ions can be generated electrochemically during the ESI process.

Typical examples of analytes ionized as radical ions during ESI are: fullerenes (C60), organometallic compounds and metallocenes, aromatic or highly conjugated compounds (for example, PAHs):

The presence of cations like Ag⁺ can be a promoting factor:

\[
\text{PAH} + \text{Ag}(\text{l}) \rightarrow \text{PAH}^{+} + \text{Ag}(0)
\]
Peptide and protein spectra in ESI-MS

Peptide and proteins are some of the molecules that most benefited from the introduction of ESI-MS: multi-charging enables MS detection of large biomolecules (high MW) at relatively low m/z ratios.

In the case of peptides, ions having up to up to +6/+7 charge can be sometimes observed.

The presence of charges (z) higher than 1 reflects on the distance between isotopic peaks, that is given by (1/z):

\[
\text{m/z units} = \frac{1}{z}
\]

Pyroglutamic acid

\[
\text{Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH}_2
\]

Bombésine

\[
(M+2H)^{2+}
\]

\[
M_{\text{mono}} = 1618.8 \text{ Da}
\]
In the case of proteins, several charge states are observed, as shown in the ESI-MS spectrum of myoglobin:

The peak m/z ratios and intensity distribution provide very important information on the protein structure.
m/z ratios relevant to adjacent peaks in the multi-charge spectrum can be used to calculate the protein molecular weight and the charge state for each peak:

\[ p1 = \frac{Mr + z1}{z1} \]
\[ p2 = \frac{Mr + (z1 - 1)}{(z1 - 1)} \]

1542 \( z1 = Mr + z1 \)
1696 \( (z1 - 1) = Mr + (z1 - 1) \)

\[ Mr = 16,951 \text{ Da for } z1 = 11. \]

Note that the distance between isotopic peaks for charge states exceeding +7 is too short (e.g. 1 Da/8 = 0.125 Th) to enable their distinction (and thus the direct estimation of the charge state) unless very high resolving powers are available.
Special programs are available to perform the MW and charge state calculation iteratively, for all the couples of adjacent peaks in multi-charge spectra. Such a procedure is known as deconvolution:

A virtual spectrum in the mass (not m/z) domain is finally obtained:

Even multi-protein multi-charge spectra can be deconvolved by some programs (e.g. MagTran).
The distribution of intensity in its multi-charge spectrum reflects the tertiary structure of a protein: lower and fewer charge states will be available for a protein molecule in a folded (native) conformation. Progressive structure unfolding leads to a shift of the multi-charge spectrum towards lower m/z ratios (higher charges):
Actually the acidity of the protein solution plays a fundamental role in determining the charge state distribution.

ESI-MS spectra for $10^{-5}$ M cytochrome C in different aqueous solutions.

Note that two conformationally different protein populations are present at intermediate pH.
Simple stable ions such as the alkali metal ions $M^+$ are transferred to the gas phase without change (typically by Ion Evaporation). They usually enter the gas phase as solvated ions, $M^+(H_2O)_m$, when the solvent is water.

The solvent molecules can be removed by heating, assistant gas or collision-activated decomposition (CAD) of the ion in the interface to the mass spectrometer, so that the naked ions $M^+$ can be observed.

Species not ionizable in solution but having polar groups, such as sugars and glycols, are usually cationised (i.e. form strongly bonded complexes) by stable ions like Na$^+$ and K$^+$.

Analytes that are protonated bases (positive ion mode) or deprotonated acids (negative ion mode) might show differences between solution and gas phase ions.

The changes may occur either in the transition of the ion from the charged droplet to the gas phase or later in the gas phase, due to ion–molecule reactions.
A solution additive with high gas phase basicity may compete for charge with the analyte, thus decreasing its charge.

In this example, addition of increasing amounts of diethyl-amine lowers the charge states of cytochrome C:
Charge reduction due to collision-activated decomposition

When solvated ions generated in the ESI interface cross the nozzle represented by a hole in the mass plate (counter-electrode), several phenomena occur, strongly depending on the instrument configuration.

Three configurations are usually adopted for longitudinal ESI commonly used nowadays (i.e. without heated curtain gas):

1) only plate (grounded) with a nozzle

2) metal capillary (grounded) passing through a hole in the plate, co-axial with the spray capillary

3) glass capillary passing through the plate, covered with a metal layers only at the two ends
Whatever the configuration, complex gas dynamics have to be considered for the ESI source gas, usually $\text{N}_2$, when flowing through the nozzle located on the plate or at the capillary end.

The pressure difference (1 atm outside vs. few torr inside) is high enough to make the gas expand at the sound speed (Mach 1) near the nozzle.

Gas expansion waves reach the so-called jet boundary where they are reflected as compression waves towards the flow direction: the flow becomes supersonic (Mach > 1) and the so-called silent zone is created.

Due to supersonic expansion the local gas pressure becomes lower than ambient pressure thus the expansion is recompressed by a system of oblique shock waves, delimiting the so-called barrel shock.
The barrel shock changes the flow direction and may reduce the Mach number to subsonic values: the cross sectional area decreases, the pressure increases and, finally, at the so-called Mach disk, where compression waves intersect, the initial pressure is re-established.

In ESI mass spectrometers a skimmer can be placed slightly before/beyond the Mach disk:

An electric potential is also applied between the nozzle (or the capillary end) and the skimmer: neutrals accompanying ESI-generated ions are partly discarded.
Sometimes, the nozzle or the capillary tip axis are placed off the skimmer axis, so that neutrals, unsensitive to the electric potential, impact against the skimmer lateral surface, whereas ions are deviated towards the skimmer hole:

Gas expansion and, if a capillary is used at the mass spectrometer entrance, heating can be exploited to promote ion desolvation at this stage.

However, besides desolvation, collisions between ions and neutrals can lead to charge reduction and/or fragmentation. The phenomenon is known as collision-activated decomposition (CAD).

Actually, the collisions can be enhanced if the potential difference between nozzle/capillary and skimmer is increased (In Source-Collisional Induced Dissociation, IS-CID).
**Supercharging**

In the absence of other factors, the surface tension of the electrospray droplet late in the desolvation process is a significant factor in determining the overall analyte charge:

The plot of average charge observed for cytochrome C as a function of the surface tension of additives used in its aqueous solution shows that supercharging occurs on the protein when m-Nitrobenzyl-alcohol is used as additive.
Summary of processes influencing protein charging in ESI-MS
ESI-MS study of non-covalently bound complexes

If the experimental conditions (spray voltage, desolvation temperatures, voltages in the ion-transfer region of the MS spectrometer) are chosen properly, ESI can enable the preservation of non covalent complexes along the path between solution phase and gas phase:

**Bulk Solution Phase**
Solvated protein complex with dissociation constant $K_D$

**Solvated Phase**
Desolvation of highly charged droplet/ion by activation.

**Gas Phase**
Stability of ion for intact protein complex may or may not reflect $K_D$. 
Effect of cone voltage

The increase of cone voltage in orthogonal ESI (or, equivalently, of capillary-skimmer voltage in longitudinal ESI) can disrupt non covalent complexes.

In the case of heme-myoglobin complex (hmyo) disruption, with detection of apomyoglobin (amyo) ions, begins between 50 and 110 V:
Effect of desolvation temperatures

If the desolvation temperatures are too high non-covalent complexes may be disrupted.

In this example, ESI-MS spectra are compared for streptavidin (MW 52800 Da):

At 100°C large peaks, arising from convolution of mass peaks for different protein multimers at high charge states, are observed. Raising the temperature to 200 °C leads to single-protein gas phase ions.
A special case: observation of duplex and triplex complexes in ESI-MS spectra of oligo-DNA/PNA mixtures after prolonged interaction

Oligo-DNA dpA_{10} (ADN)

Peptide Nucleic Acid, PNA-T_{10} (APN)

after 10 min

after 3 hours
Quantitative aspects in ESI-MS

The ESI-MS signal for a single analyte usually deviates from linearity for concentrations higher than $10^{-4}/10^{-3}$ M:

The main reasons for this behaviour are:

- limited amount of excess charge
- limited space on droplet surface
- ion suppression

![Graphs showing deviation due to background interference (ionic noise)](image)

![Graph showing selected ion current against concentration for different quats](image)
The ionization yield (and then the MS response) can be very different even for structurally similar analytes:

The ESI-MS signals for a series of tri-peptides differing just for the last aminoacid appear to be correlated to hydrophobicity: the higher the latter the higher the signal.

A plot of relative MS responses vs relative retention times in isocratic RPC (related to hydrophobicity) confirms the hypothesis.
A further factor that has to be taken into great account for quantitative determinations in ESI-MS is competition for ionization:

the ESI-MS response for an analyte co-eluting with one or several others (especially when complex mixtures are directly infused into the MS spectrometer) can be very different from that obtained for the same compound as the unique solute.

In the same way, variations in the mobile-phase or solution composition can greatly influence the response.

Last, but not least, fluctuations in spray voltage or nebulising gas flow rates can reduce reproducibility and then affect calibration precision.

Internal standardization, eventually with isotopically labelled analogues, can greatly improve analytical performances in quantitative ESI-MS.
Scaling down ESI-MS: from conventional to nano-ESI

Since the early stages of ESI-MS development it was clear that lower flow rates would have improved ionization efficiency.

A lower radius can be predicted for the initial droplets at lower flow rates, thus subsequent Coulomb fission is favoured.

\[ R \approx \left( \frac{V_f \varepsilon}{K} \right)^{1/3} \]

Indeed, a decrease in MS response is observed when the flow rate is increased, unless the ESI process is assisted by higher gas flow rates:
Actually a compensation occurs up to flow rates of 50-100 $\mu$L/min, due to enhanced analyte transport into the interface.

When analyte flow rates are higher than 200 $\mu$L/min even pneumatic assistance is unable to guarantee a stable spray.

The requirement of low flow rates can be in contrast with the optimal flow rates for liquid chromatography, then two possible strategies have to be implemented:

✓ flow splitting

✓ scaling down
Flow splitting is usually adopted for liquid chromatography performed with conventional columns, i.e. having internal diameters greater than 2.1 mm. No splitting is usually required for narrowbore (2.1 mm), microbore (1 mm) and capillary columns (0.3 mm).
Nano-LC-ESI-MS

Columns for Nano-LC have internal diameters lower than 100 μm and are operated at flow rates lower than 1 μL/min, obtained with special pumps or splitting devices:

In this case the ESI interface is actually a metal (or internally metal-coated) microsized capillary
Interfacing between the LC column and the capillary occurs through a conducting union, to which high voltage is applied:

![Diagram of conducting union with labeled parts]

A lower onset voltage can be predicted for Nano-ESI, due to the lower capillary radius:

$$V \approx \sqrt{\frac{\varepsilon_0 \cos 49}{2 \varepsilon_0}} \cdot \ln \left(\frac{4d}{r}\right)$$

Actually, also the capillary-plate distance $d$ is lowered (from 10-20 to 1-2 mm), in order to minimise the increase of the $4d/r$ term in the onset voltage equation:

![Diagram of nitrogen flow and MS interface]
## Numerical comparison between conventional and nano-ESI

<table>
<thead>
<tr>
<th>Electrospray parameters</th>
<th>NanoES</th>
<th>Conventional ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary</td>
<td>1-3µm</td>
<td>&gt;100µm</td>
</tr>
<tr>
<td>Flow</td>
<td>10-40nL/min.</td>
<td>&gt;500nL/min.</td>
</tr>
<tr>
<td>ES voltages</td>
<td>300-700V</td>
<td>&gt;2500V</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Droplets</th>
<th>NanoES</th>
<th>Conventional ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius</td>
<td>50 - 200nm</td>
<td>1000 - 2000nm</td>
</tr>
<tr>
<td>Volume</td>
<td>5 \cdot 10^{-13} - 4 \cdot 10^{-12} µL</td>
<td>4 \cdot 10^{-9} - 3 \cdot 10^{-8} µL</td>
</tr>
<tr>
<td>Analyte molecules per droplet (at 1pmol/µL)</td>
<td>0.3 - 2.5</td>
<td>2.5 \cdot 10^3 - 1.9 \cdot 10^4</td>
</tr>
</tbody>
</table>
Gain in Analysis Time in Nano-ESI-MS

The possibility of using extremely low flow rates (usually few tens of nL) leads to a remarkable increase in analysis time with nano-ESI-MS.

As a consequence long infusion times (implying the possibility of performing several MS, MS/MS or MS^n experiments) can be reached even with low analyte amounts:

1.5 µL of sample loaded in a long NanoESI capillary at a flow rate 18 nL/min

SIM: m/z 818.6, ESI voltage: 400V, Backing pressure: approx. 2 atm
Features of Nano-ESI-MS

✓ Flow rates of 10-40 nL/min (25-100 min analysis time)

✓ Sample volumes down to 300 nL

✓ No pneumatic assistance

✓ Near 100% sample utilization

✓ Minimal instrument contamination

✓ Zero sample cross contamination

✓ Spray from 0% to 100% aqueous solvents

✓ Ionization efficiencies of 50-60% (vs 0.01% in conventional ESI)

✓ Detection limits down to $10^{-15}/10^{-18}$ moles (femto/atto-moles)
The ESI advantages

😊 ESI is the purest form of transfer of ions to the gas-phase (little if any extra internal energy is given to the ions), capable of generating even non-covalent complexes in the gas phase.

😊 ESI enables ion transfer from solution to the gas phase for a large range of ion types: from singly charged electrolytes to multiply-protonated peptides and proteins up to 100,000 Da and multiply deprotonated negatively charged nucleic acids.

😊 Solutions used for transfer of ions to the gas phase are the same as for conventional wet chemistry (wide range of solvents acceptable).

😊 Sample introduction through LC, CE, FIA, and direct infusion.

😊 Compatible virtually with all the currently adopted mass analyzers.

😊 Good sensitivity with lower limits in the femtomole to low picomole ranges.

😊 Multiple charging allows for analysis of high mass ions with a relatively low m/z range instrument.
The ESI disadvantages

- The presence of salts can reduce sensitivity (salt ions are much more easily formed that analyte ions)
- Complex mixtures can reduce sensitivity (due to competition for ionization)
- Simultaneous mixture analysis (e.g. without separation) can be poor
- Multiple charging can be confusing especially in mixture analysis (e.g. multi-charge spectra for mixtures of several proteins)
- Sample and solvent purity is important (use of LC-MS grade solvents is recommended to reduce ionic noise)
General ESI – MS applications

- Molecular weight determination and structure analysis (e.g. proteins, peptides, nucleic acids, ….)

- Analysis of conformational structures (e.g. secondary and tertiary structures of proteins)

- Studies of non-covalent molecular interactions (e.g. antibody-antigen, receptor-ligand, enzyme-substrate, …)

  - Gas-phase chemistry

  - Inorganic-organometallic chemistry

    - Elemental speciation

  ✓ Trace analysis
Comparison between ESI and other ionization approaches
Desorption Electrospray Ionization (DESI)

DESI, developed by R.G. Cooks in 2004, is carried out by directing electrosprayed charged droplets and ions of solvent onto the surface to be analyzed. The impact of charged particles produces gas phase ions from the material originally deposited on the surface.
In the original experiment, the sample solution was deposited on a Teflon surface and a methanol-water (1:1 v/v) mixture containing 1% acetic acid (or a 0.1% aqueous acetic acid solution) was sprayed at a flow rate of 3 to 15 µL/min under the influence of a high (4 kV) voltage. The nominal linear velocity of the nebulizing gas was set to 350 m/s.

Using multi-spot target surfaces, analyses on several analytes can be performed, sequentially, by DESI:
Angles $\alpha$ and $\beta$ and distances $d_1$ and $d_2$ can be varied by moving and rotating the sample stage:

Experimental tests show that:

- High incident angles (70-90°) and short $d_1$ distances (1-3 mm) are optimal for high MW compounds (proteins, peptides, oligo-saccharides).
- Low $\alpha$ angles (35-50°) and longer $d_1$ distances (7-10 mm) are better suited for analysis of low MW species (diazo dyes, steroids, caffeine, nitro-aromatics).

<table>
<thead>
<tr>
<th>$\alpha$</th>
<th>Incident angle</th>
<th>$0-90^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
<td>Collection angle</td>
<td>$5-10^\circ$</td>
</tr>
<tr>
<td>$d_1$</td>
<td>Tip to surface distance</td>
<td>$1-10$ mm</td>
</tr>
<tr>
<td>$d_2$</td>
<td>MS inlet to surface distance</td>
<td>$0-2$ mm</td>
</tr>
</tbody>
</table>
Mechanisms for DESI

Two different mechanisms have been proposed for DESI, according to the molecular weight of the analytes:

1) High MW compounds (ESI-like)

High MW molecules, like proteins and peptides, show ESI-like spectra after DESI, i.e. multiply charged ions. This observation suggests that:

- a charged droplet hits the sample, spreading over a diameter greater than its original one, dissolves the peptide or protein and bounces
- multiple charge exchanges occur between solvent and analyte within the droplet, while it is travelling towards the mass spectrometer inlet
- desolvation of the droplet occurs in the ion transfer line of the mass spectrometer, thus almost desolvated, multi-charge ions can enter the mass analyser.
2) Low MW compounds (APCI-like)

In this case ionization occurs by charge transfer via an electron or a proton. There are three possibilities for the charge transfer:

- between a solvated anion (A) and an analyte (S) molecule on the surface:
  \[ A_{(aq)}^- + S_{(s)} \rightarrow A_{(aq)} + S_{(s)}^- \]

- between a gas phase anion and an analyte molecule on the surface:
  \[ A_{(g)}^- + S_{(s)} \rightarrow A_{(g)} + S_{(s)}^- \]

  In this case the ion solvent is evaporated before reaching the sample surface; this is achieved when the spray-to-surface distance is large.

- between a gas phase anion and a gas phase analyte molecule:
  \[ A_{(g)}^- + S_{(g)} \rightarrow A_{(g)} + S_{(g)}^- \]

  This occurs when the analyte has a high vapor pressure.
DESI for in vivo sampling of living tissue surfaces

One of the fundamental advantages of DESI is the possibility of making analysis on real samples with minimal, if any, pretreatment, under ambient conditions.

Even in vivo measurements can be made!

One of the most astonishing applications of DESI is the analysis of proteins or drugs on the surface of a finger tip:

A water/ethanol spray is directed onto the finger of a person who has taken a specific drug.
About 40 min after the patient has taken a tablet of Loretadine, an anti-staminic, the molecule becomes detectable directly on the skin or in saliva and its surface concentration remains above the detection limit for another 50 min:

This example is representative of the novel applications of MS once its vacuum constraints are lifted.
DESI analysis of biological fluids

DESI mass spectrum of dried, 2-mL, raw urine spots on paper, sprayed with a 1:1 methanol:water mixture containing 1% acetic acid:

The product ion (MS/MS) spectrum identifies one of the minor components, with m/z = 214, as L-aspartyl-4-phosphate.
**DESI analysis of chemical weapons (nerve gases)**

MS/MS spectra obtained for Diethyl-phosphoramidate (m/z 182, CE: 10 V), Diisopropyl-ethyl-phosphate (m/z 211, CE: 7 V) and Tabun (m/z 163, CE: 7 V) during the DESI analysis of a SPME fiber previously exposed to the headspace of a Dacron swab spiked with munitions-grade Tabun:
DESI analysis of explosives

DESI can be successfully applied to test the presence of explosive traces on different samples:

TNT: trinitro-toluene
RDX: hexahydro-trinitro-1,3,5-triazine
HMX: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine:
PETN: pentaerythritol tetranitrate
C(CH$_2$O-NO$_2$)$_4$
TATP: triacetone-triperoxide

<table>
<thead>
<tr>
<th>Explosives</th>
<th>Ions observed</th>
<th>LDD (pg)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>M$^-$</td>
<td>10</td>
<td>Plastic</td>
</tr>
<tr>
<td></td>
<td>(M - NO)$^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDX</td>
<td>(M + H)$^+$</td>
<td>1</td>
<td>Paper</td>
</tr>
<tr>
<td>RDX</td>
<td>(M + A)$^-$</td>
<td>10</td>
<td>Paper</td>
</tr>
<tr>
<td></td>
<td>(M - H)$^-$</td>
<td>100</td>
<td>Metal</td>
</tr>
<tr>
<td>HMX</td>
<td>(M + A)$^-$</td>
<td>10</td>
<td>Metal</td>
</tr>
<tr>
<td>PETN</td>
<td>(M - H)$^-$</td>
<td>100</td>
<td>Paper</td>
</tr>
<tr>
<td></td>
<td>(M + A)$^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TATP</td>
<td>(M + Na)$^+$</td>
<td>1000</td>
<td>Paper</td>
</tr>
</tbody>
</table>

A, anion: Cl$^-$, acetate, trifluoro-acetate, nitrate
Surface MS imaging by DESI

By scanning the charged-droplet beam across the surface of a sample in a two-dimensional array of predefined points, molecular images can be constructed by plotting the intensity of one or more of the DESI-MS signals derived from the surface as a function of position in the array.

Motion pattern of the surface during Imaging DESI-MS acquisitions

The small arrows indicate the verse of the spray directed onto the surface

Images from a 10 μm sagittal rat brain section

optical microscopy

DESI-MS (m/z 772)
Survey of possible applications for DESI-MS

✓ Examination of native surfaces for forensic science (drugs, natural compounds)

✓ Examination of biological surfaces (eventually with imaging and in vivo), both animal and vegetal (i.e. pigments, essential oils in plant material)

✓ High-throughput examination of solutions after transfer onto a surface (metabolomics)

✓ Analysis of explosives and chemical weapons on surfaces (military security, anti-terrorism).
A comparison between the main Desorption Ionization techniques in Mass Spectrometry

<table>
<thead>
<tr>
<th>Ionization Method</th>
<th>Energy Source</th>
<th>Flux</th>
<th>Matrix</th>
<th>Mass Analyzer</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static secondary ion MS (SIMS)</td>
<td>keV ions</td>
<td>$10^{-10}$ A cm$^{-2}$</td>
<td>None, solid</td>
<td>Any</td>
<td>Surface sensitive, nondestructive, low signal</td>
</tr>
<tr>
<td>Liquid SIMS</td>
<td>keV ions</td>
<td>$10^{-6}$ A cm$^{-2}$</td>
<td>Liquid</td>
<td>Any</td>
<td>Higher, longer lasting signal</td>
</tr>
<tr>
<td>Fast atom bombardment (FAB)</td>
<td>keV atoms</td>
<td>$10^{13}$ atoms cm$^{-2}$ s$^{-1}$</td>
<td>Liquid</td>
<td>Any</td>
<td>High, long-lasting signal, high background</td>
</tr>
<tr>
<td>Plasma desorption (PD)</td>
<td>MeV ions</td>
<td>$10^3$ particles cm$^{-2}$ s$^{-1}$</td>
<td>Nitrocellulose</td>
<td>Time-of-flight</td>
<td>High ionization efficiency, low signals</td>
</tr>
<tr>
<td>Laser desorption (LD) and matrix-assisted laser desorption (MALDI)</td>
<td>Photons</td>
<td>$\geq 10^6$ watt cm$^{-2}$</td>
<td>Solid matrix absorbs radiation in MALDI</td>
<td>Usually time-of-flight</td>
<td>CW lasers can cause thermal degradation</td>
</tr>
</tbody>
</table>
## A comparison between Molecular Ionization Techniques

<table>
<thead>
<tr>
<th>Ionization Method</th>
<th>Ion Type</th>
<th>Sample Type</th>
<th>Separation Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI</td>
<td>$M^{+*}$, fragments</td>
<td>Nonpolar and some polar organic compounds</td>
<td>GC</td>
</tr>
<tr>
<td>CI</td>
<td>$[M + H]^+$, $[M - H]^-$, $M^-$</td>
<td>Nonpolar and some polar organic compounds</td>
<td>GC</td>
</tr>
<tr>
<td>Thermospray</td>
<td>$[M + H]^+$, $[M - H]^-$,</td>
<td>Polar compounds</td>
<td>LC</td>
</tr>
<tr>
<td></td>
<td>$[M + NH_4]^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAB</td>
<td>$[M + H]^+$, $[M - H]^-$</td>
<td>Peptides, proteins, lipids, carbohydrates, oligosaccharides, nucleotides, oligonucleotides</td>
<td>LC, CE</td>
</tr>
<tr>
<td>APCI</td>
<td>$[M + H]^+$, $[M - H]^-$</td>
<td>Polar compounds, drugs</td>
<td>LC</td>
</tr>
<tr>
<td>ESI</td>
<td>$[M + nH]^{n-}$, $[M - nH]^{n-}$</td>
<td>Peptides, proteins, lipids, carbohydrates, oligosaccharides, oligonucleotides</td>
<td>LC, CE</td>
</tr>
<tr>
<td>MALDI</td>
<td>$[M + H]^+$, $[M - H]^-$</td>
<td>Peptides, proteins, lipids, carbohydrates, oligosaccharides, oligonucleotides</td>
<td>LC, CE</td>
</tr>
</tbody>
</table>
Atmospheric Pressure Photo-Ionization (APPI)

Following the same principle of photo-ionization detectors (PID) used in GC, APPI has been introduced in 2000 to overcome the limitations of ESI and APCI towards specific classes of analytes.

It is based on the UV radiation-induced ionization of analyte molecules after nebulization at high temperature.

UV radiation is usually generated by a Krypton lamp.
The energies of radiation emitted by a Kr lamp (10.0 and 10.6 eV) are able to ionize several classes of organic compounds but not potentially interfering compounds like atmospheric gases:

\[
\begin{align*}
N_2 & \quad – 15.58 \text{ eV} \\
O_2 & \quad – 12.07 \text{ eV} \\
H_2O & \quad – 12.62 \text{ eV} \\
CO_2 & \quad – 13.78 \text{ eV}
\end{align*}
\]

or typical LC solvents:

<table>
<thead>
<tr>
<th>PhotoMate™ lamp</th>
<th>Krypton 10.0 eV, 10.6 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionization Potentials (IP)</td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>7.4 eV</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>7.8 eV</td>
</tr>
<tr>
<td>Caffeine</td>
<td>8.0 eV</td>
</tr>
<tr>
<td>4-Nitrotoluene</td>
<td>9.5 eV</td>
</tr>
<tr>
<td>2,4,6-Trinitrotoluene</td>
<td>10.59 eV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent Ionization Potentials</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>10.85 eV</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>12.19 eV</td>
</tr>
<tr>
<td>Water</td>
<td>12.61 eV</td>
</tr>
</tbody>
</table>
Two main mechanisms can be invoked to explain the APPI process:

**Direct APPI**

Ionization may occur directly on the analyte molecule \((M)\), leading to the molecular ion:

\[
M + h\nu \rightarrow M^+ + e^-
\]

Alternatively, solvent \((S)\) molecules can act as mediators:

\[
M + S + h\nu \rightarrow [M + H]^+ + [S - H]^- 
\]

and quasi-molecular ions can be generated.
Dopant-assisted APPI

A dopant (D) is added to the LC effluent and co-nebulized with the analyte. It is chosen to be ionized preferentially by UV radiation.

Afterwards, its radical cations can ionize analyte molecules, leading to molecular or quasi-molecular ions, according to the case:

\[ D^{++} + M \rightarrow M^{++} + D \]

\[ D^{++} + M \rightarrow [M + H]^+ + [D - H]^+ \]

Toluene, acetone, methoxy-benzene (anisole) and chlorobenzene are commonly used in APPI-MS as dopants.
APPI is particularly well-suited for the analysis of compounds with relatively low MW and low polarity, which are not easily ionized by ESI and APCI.

Steroids are a typical example:

Like APCI, APPI enables MS detection in conjunction with normal-phase chromatography but is not suitable for the analysis of thermolabile compounds (e.g. biomolecules) due to severe heating (350-550 °C) in the nebulizing step.