LC-MS
Τεχνολογία Εφαρμογές

Γ. Θεοδωρίδης Τμήμα Χημείας ΑΠΘ

Χαρακτηριστικά

- Η μεγαλύτερη και η πιο αναπτυσόμενη τεχνολογία ανάλυσης
- Κύριο εργαλείο βιοανάλυσης, φαρμακευτικής ανάλυσης, ανάλυσης μεταβολιτών, ρυπαντών κλπ
- Οργανο αναφοράς για πλήθος ενώσεων
- Προσφέρει δυνατότητες που δεν προσφέρει καμία άλλη τεχνολογία *(μεγάλη-σημαντική διαφορά με άλλες τεχνολογίες που δεν προσφέρουν μοναδικά πλεονεκτήματα, π.χ. SFC)*.

Introduction to Mass Spectrometry - Ionization

- General Outline of LC-MS/MS
- Ion Sources
  - Electrospray/IonSpray
  - APCI
  - APPI
  - MALDI
  - ICP
  - Others (inc FAB, FD/FI, EI/CI, TSP, PB)

Principles of Mass Spectrometry (1)

- What is a mass spectrometer?

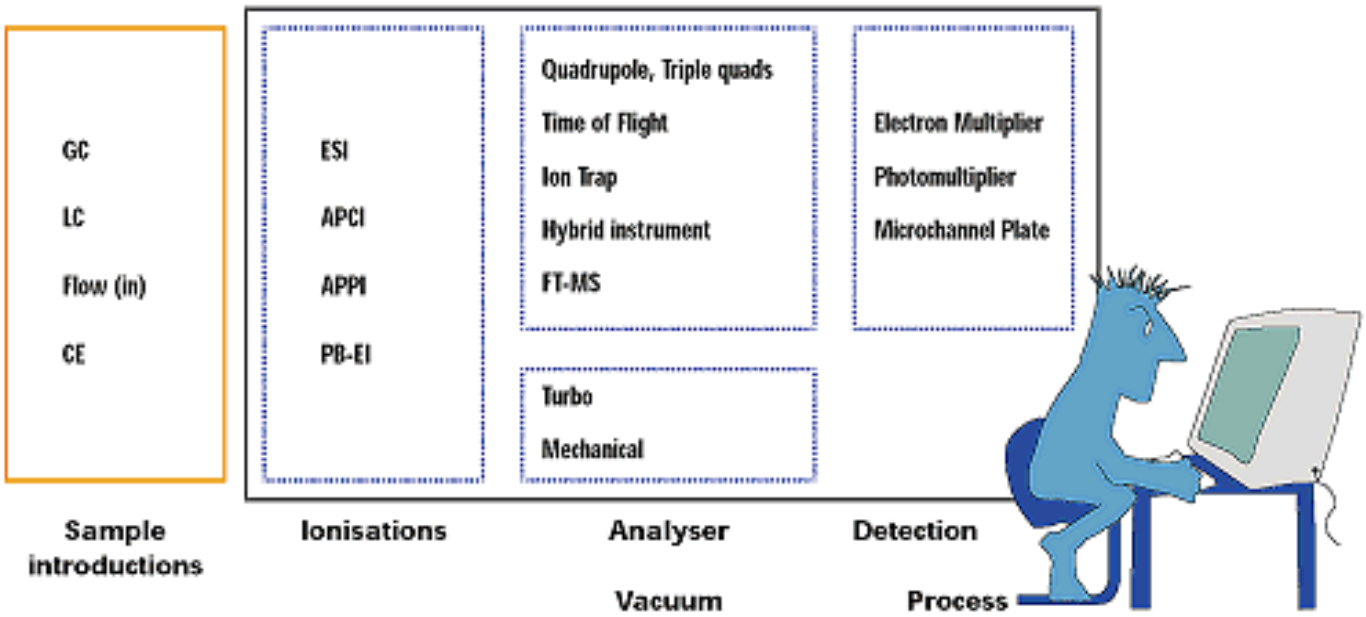
- The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z (mass to charge ratio) value.

The analyzer uses electrical or magnetic fields, or combination of both, to move the ions from the region where they are produced, to a detector, where they produce a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, it is the mass to charge ratio, and not only the mass, which is of importance. The analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield.

MS/MS is the combination of two or more MS experiments. The aim is either to get structure information by fragmenting the ions isolated during the first experiment, and/or to achieve better selectivity and sensitivity for quantitative analysis.
MS/MS is done:

- either by coupling multiple analysers (of the same or different kind)
- or, with an ion trap, by doing the various experiments within the trap

Principles of Mass Spectrometry



In addition to the analyzer, the mass spectrometer also includes

- A vacuum system
- Tools to introduce the sample (LC, GC …)
- Tools to produce the gas phase ions from the sample molecules
- Tools to fragment the ions, in order to obtain structural information, or to get more selective detection
- A detection system
- Software and computing

Principles of Mass Spectrometry

- LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionization.

- In all cases, there is the need for an interface that will eliminate the solvent and generate gas phase ions, then transferred to the optics of the mass spectrometer.

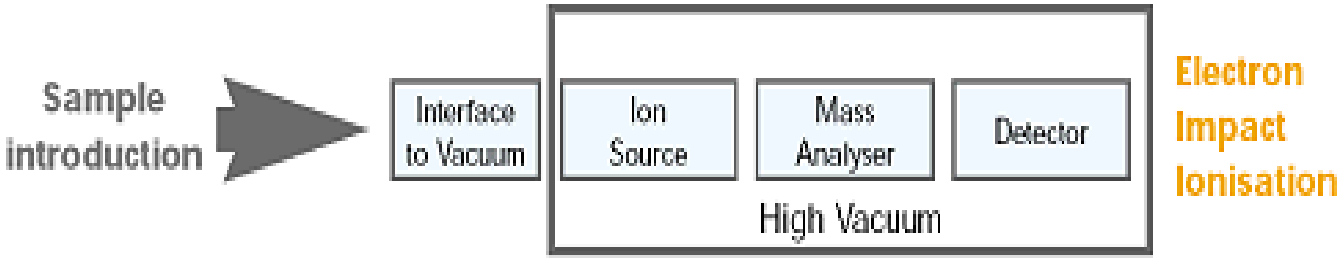
- Most instruments now have atmospheric pressure ionization (API) techniques where solvent elimination and ionization steps are combined in the source and take place at atmospheric pressure.

- When electron impact ionization (EI) is the choice, the solvent elimination and ionization steps are separate.

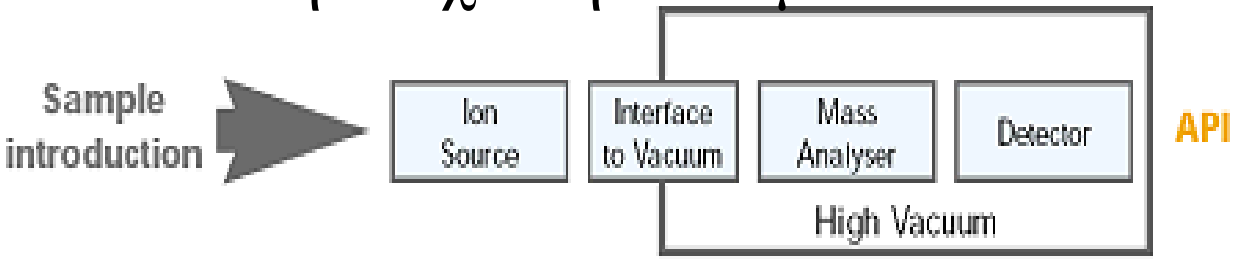
- Electron impact is of interest for molecules which do not ionize with API technique, or when an electron impact spectrum is necessary, since it provides spectral information independent of the sample introduction technique (GC or LC, or direct introduction) and instrument supplier.

Principles of Mass Spectrometry

«Σκληρή» τεχνική ιονισμού

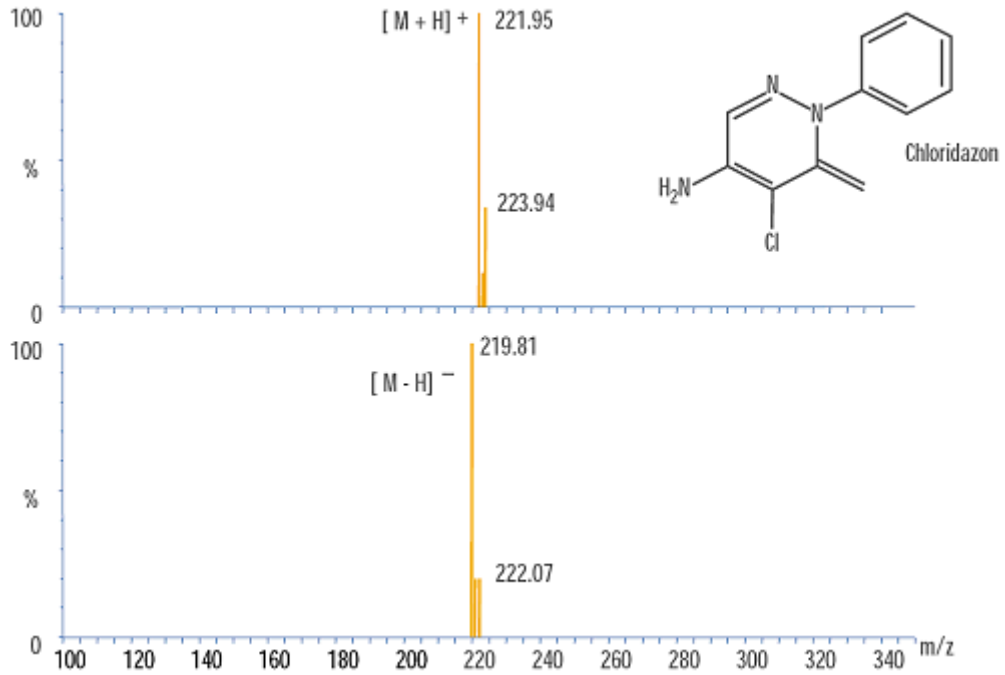


«Μαλακή» τεχνική ιονισμού



Mass Definition

The mass spectrometer measures the exact mass. Looking at the below mass spectra, the most abundant peak is at 221.95 (top) and 219.81 (bottom). These spectra are obtained with positive ionisation (top) and negative ionisation (bottom). The peaks correspond to the protonated or deprotonated molecule.



Mass Definition

If we look for the molecular mass of the chloridazon pesticide, we can find various values in the spectra

- **221.6379:** this is the average mass. It is based on the average atomic masses.
- **221.0278:** this is the exact (or monoisotopic) mass, based on the exact mass of the most abundant natural isotopes
- **221**: this is the nominal mass, calculated on the nominal mass of the most abundant isotopes

The valueobtained from the mass spectrometer is slightly different from the expected 222.0278 and 219.0278 because these spectra were obtained with a quadrupole instrument, which does not provide sufficient mass resolution and mass accuracy for obtaining the exact mass.

The next smaller peaks correspond to the ^{13}C and ^{37}Cl isotopes.

Conclusion

The application field of LC/MS is extremely large and is covered by a wide range of instruments and techniques.
Looking globally at the users, it is possible distinguish three groups, depending on how they use LC/MS.

- Users for which the main useful information from the mass spectrometer is the mass information (molecular weight or fragments). The quantitative aspect is of no or little importance.

- Users for which the main interest is getting a very selective and sensitive detection. These users are targeting specific molecules. The quantitative aspect is important, but the mass information is of secondary importance.

- Users targeting specific molecules, wanting the quantification and the confirmation of the identity. The molecular weight, and the presence of a few specific fragments which the expected abundance are as important as the sensitivity and selectivity.

LC/MS - Interfacing HPLC And MS

Introduction

Interfacing a HPLC system with a mass spectrometer is not trivial.

The difficulty is to transform a solute into a gas phase ion.
The challenge is to get rid of the solvent while maintaining adequate vacuum level in the mass spectrometer, and to generate the gas phase ions.

Since the early seventies, a number of approaches have been used.
LC/MS became really popular with the introduction of of the thermospray interface and the particle beam interface.
The next big improvement was the introduction of the electrospray and APCI techniques.
The thermospray interface is no longer available on the market, but the particle beam is still available because it it the only method to provide electron impact spectra.
Actually, the large majority of applications are done with electrospray and APCI ionisation.
New techniques like APPI (atmospheric photo ionisation), DART and DESI are now appearing.

Introduction

Electrospray and APCI are both API (atmospheric pressure ionisation) techniques. Ionisation takes place at atmospheric pressure and both are considered to be soft ionisation methods.

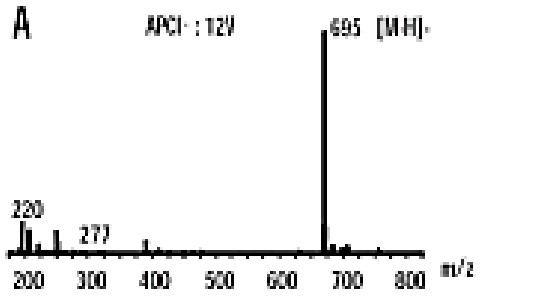
The mass spectrum provides mainly the molecular weight information, unless fragmentation techniques are used.

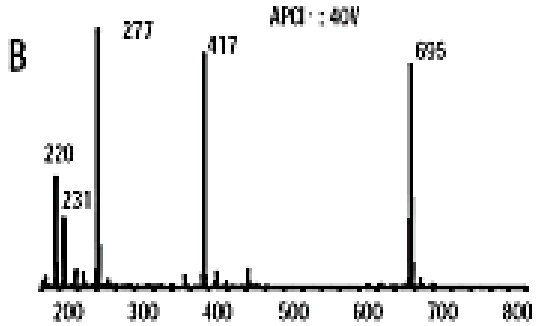
The possible fragmentation techniques are in source CID (collision induced dissociation), CID in the collision cell of a tandem type instrument, fragmentation in an ion trap.

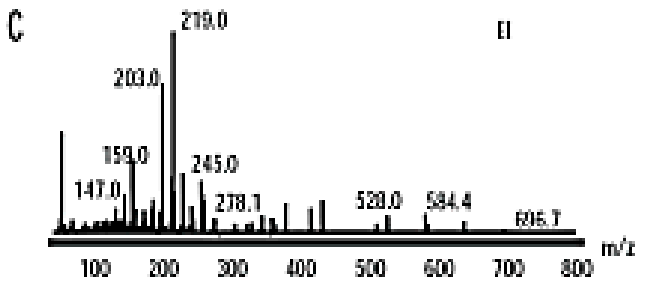
This is very different from the spectra obtained with EI (electron impact ionisation).

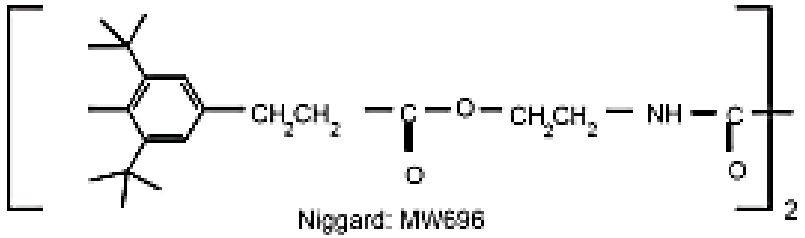
Comparison of APCI ionisation and electron impact ionisation

Niggard spectra (polymer additive), obtained by negative APCI (A), negative APCI with in source fragmentation (B), electron impact ionisation (C).



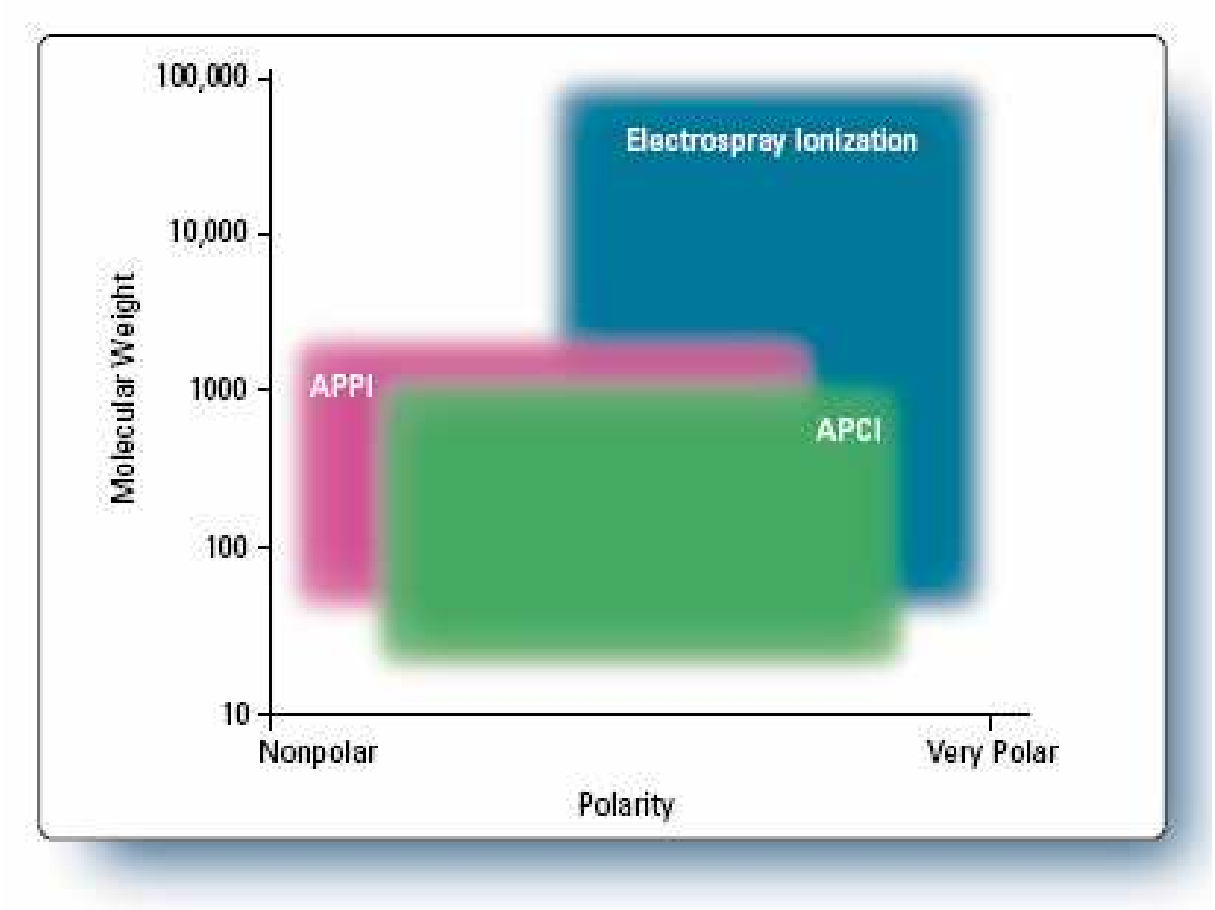






Niggard: MW696

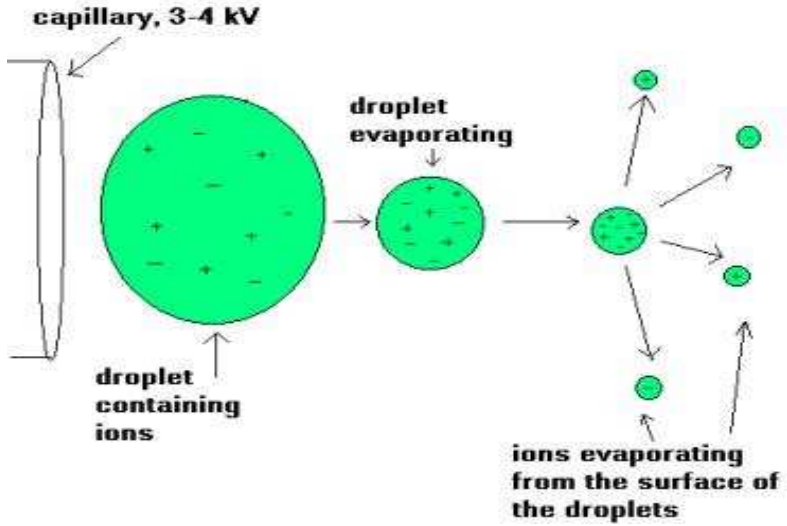
Application of various LC/MS ionisation techniques



Electrospray Ionisation - Description

The HPLC line is connected to the electrospray probe, which consists of a metallic capillary surrounded with a nitrogen flow.
A voltage is applied between the probe tip and the sampling cone. In most instruments, the voltage is applied on the capillary, while the sampling cone is held at low voltage. First step is to create a spray. At very low flow rate (a few µl/mn), the difference in potential is sufficient to create the spray.
At higher flow rate, a nitrogen flow is necessary to maintain a stable spray.

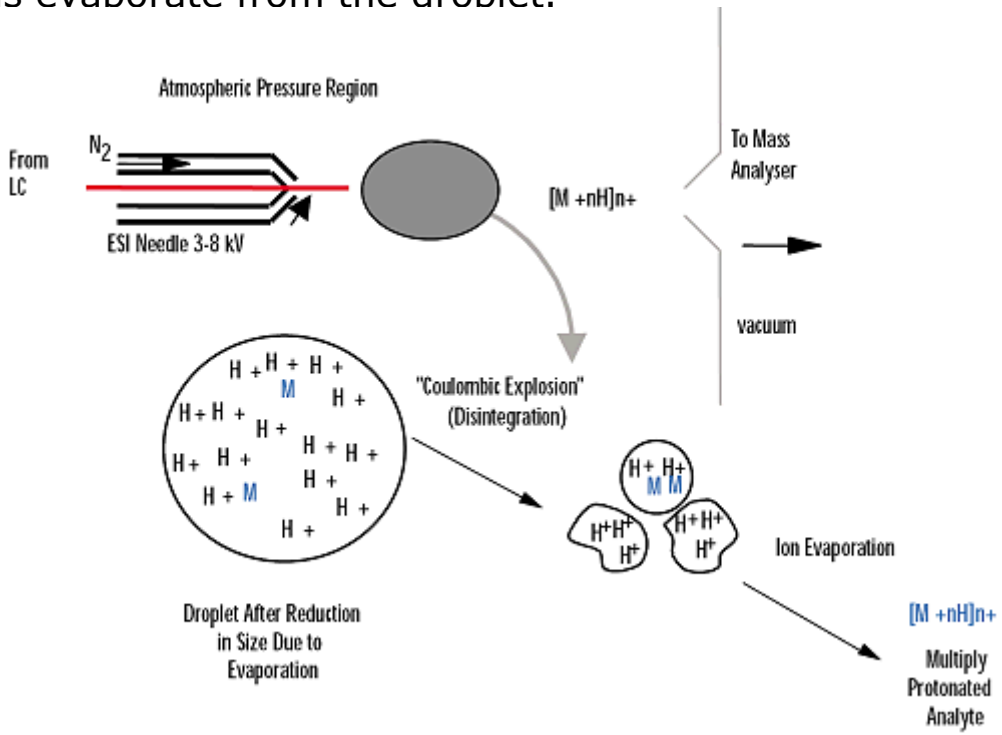
The API sources include a heating device, in order to speed up solvent evaporation. A mandatory condition to work with electrospray is that the compound of interest must be ionized in solution.



Electrospray Ionisation - Description

In the electrical field, at the tip of the capillary, the surface of the droplets containing the ionized compound will get charged, either positively or negatively, depending on the voltage polarity .

Due to the solvent evaporation, the size of the droplet reduces, and, consequently, the density of charges at the droplet surface increases. The repulsion forces between the charges increase until there is an explosion of the droplet. This process repeats until analyte ions evaporate from the droplet.



Electrospray Ionisation - Description

Typical ions produced by electrospray ionisation:	
Positive mode:	[M+H]+ protonated molecule [M+Na] +, [M+K] + … adducts [M+CH3CN+H] + protonated, + solvent adducts
Negative mode:	[M-H] - deprotonated molecule [M+HCOO -] -, … adducts

Electrospray Ionisation - Operating conditions

Flow rate: the best sensitivity is achieved at low flow rate. Working at 1 ml/mn or even higher is technically possible, but may cause a reduction in the signal to noise ratio.

Eluent pH: the mobile phase should have a pH such that the analytes will be ionized. An acidic mobile phase is suitable for the analysis of basic compounds, using positive ESI, while a basic pH will be chosen for analyzing acidic molecules. However, some exceptions exist to this general rule: positive ESI of basic compounds with a high pH mobile phase has been published.

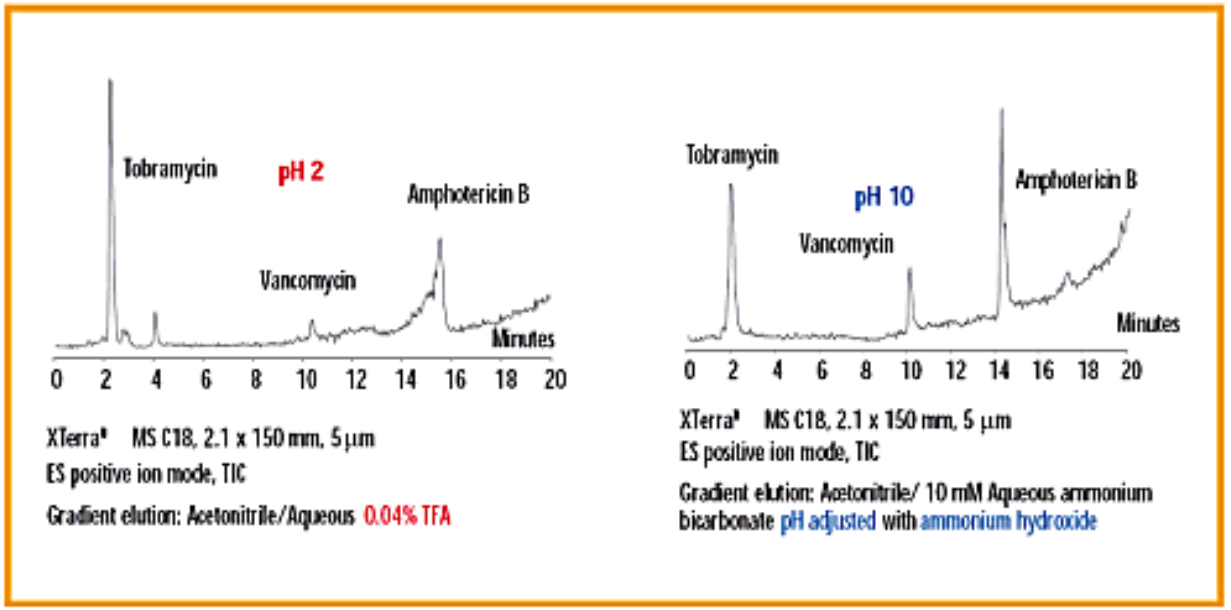
Buffers: volatile buffers are to preferred for routine use. Operating the instrument with non-volatile buffers such as phosphate is technically possible, but the salt deposit in the source will have to be removed periodically. The concentration of the buffer, or acid or base used to adjust/control the pH should be as low as possible. If not, competition between analyte and electrolyte ions for conversion to gas-phase ions decreases the analyte response.This can be explained as follows: if a species is in large excess, it will cover the droplet surface and prevent other ions to access the surface, and thus to evaporate. A species in large excess will also catch all charges available and prevent the ionisation of other molecules present at much lower concentration.

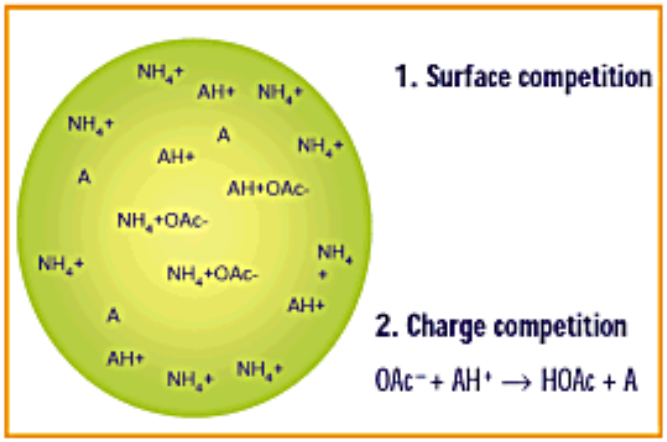
Electrospray Ionisation - Operating conditions

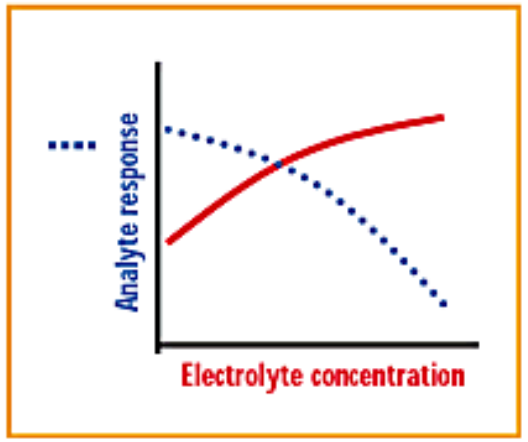
Ion pairing agents (sodium octanesulfonates…..) : these molecules have surfactant properties. The presence of surfactants in the mobile phase impacts the spray formation and droplet evaporation. There is also a surface competition mechanism phenomenon.

Matrix effects: when the sample contains high concentration of salts, or an excess of another analyte that can ionise in the operating condition, there might be a competition effect in the ionisation. This is called "ion suppression". The chromatographic separation must be developed to remove this effect, at least when doing quantitative analysis.

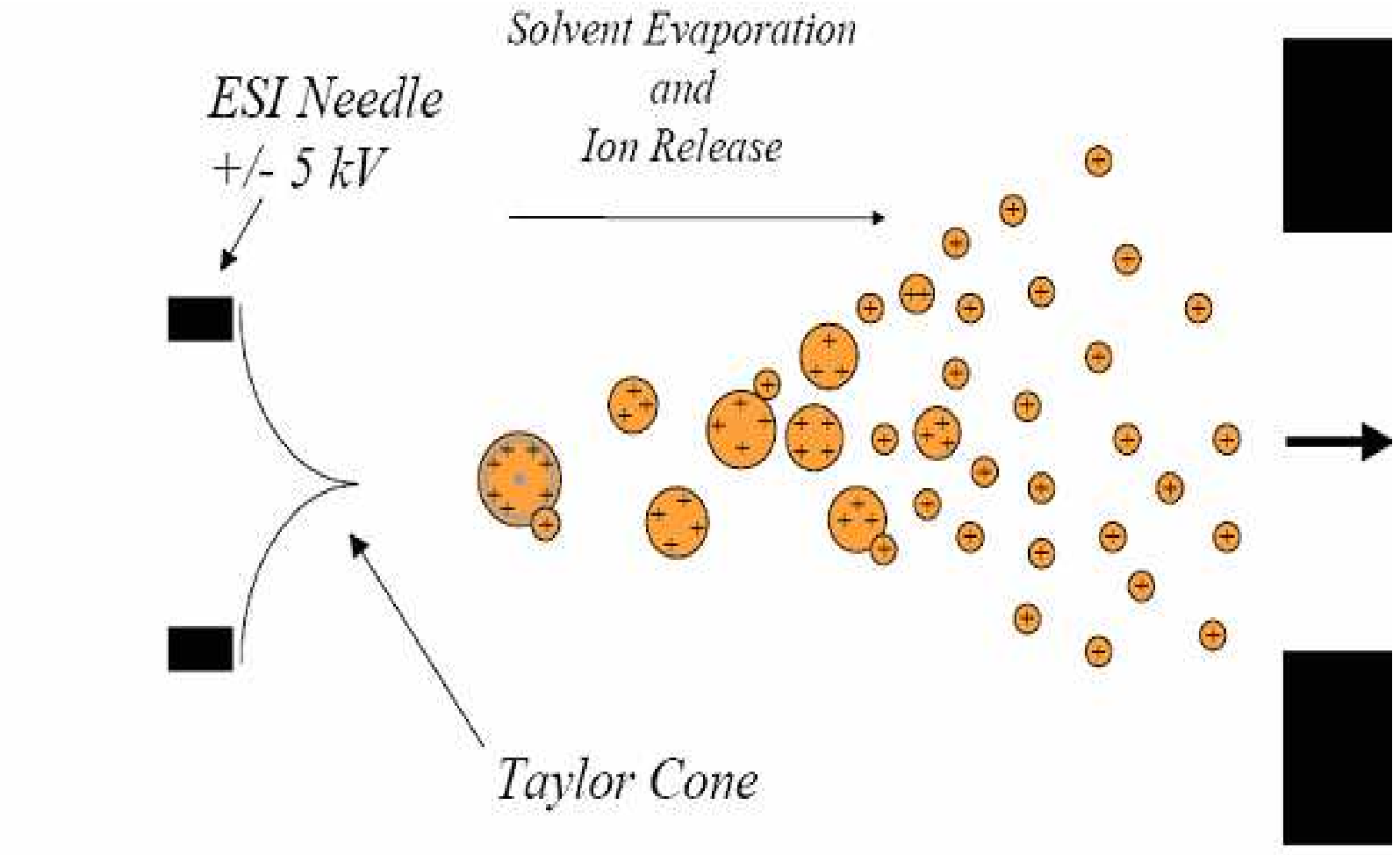
Electrospray Ionisation - pH effects, surface competition and suppression







Electrospray – Basic Layout



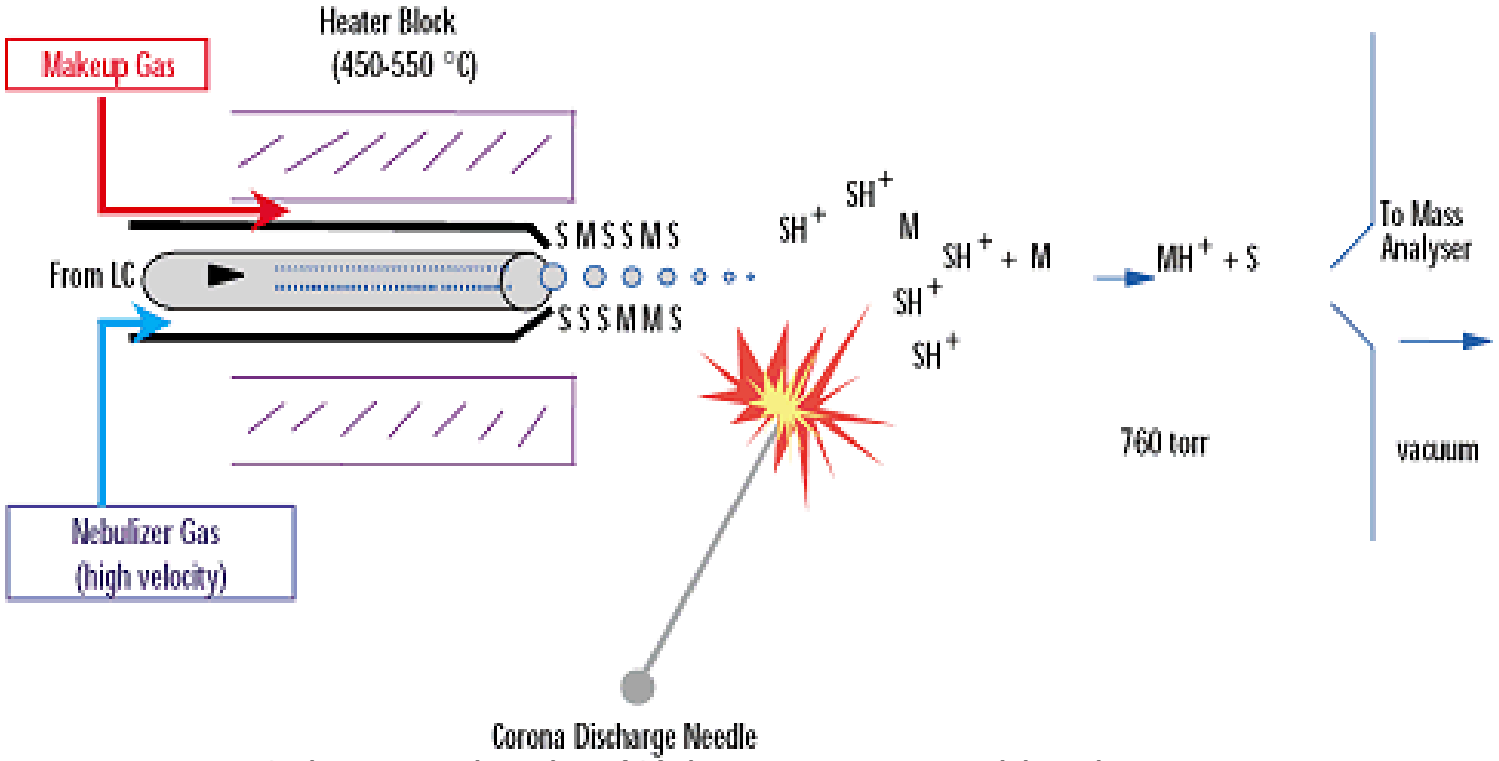
IonSpray Features

- High Sensitivity for biological compounds
- Produces molecular ion with no thermal degradation
- Low sample consumption
- Compatible with a variety of separation methods (eg LC, CE)
- Molecular weight dimensions into the kDa mass range
- Fully articulatable for optimum performance
- Flow rates from 0.1 μl/min to ~40 μl/min

LC/MS Buffers and Additives Summary

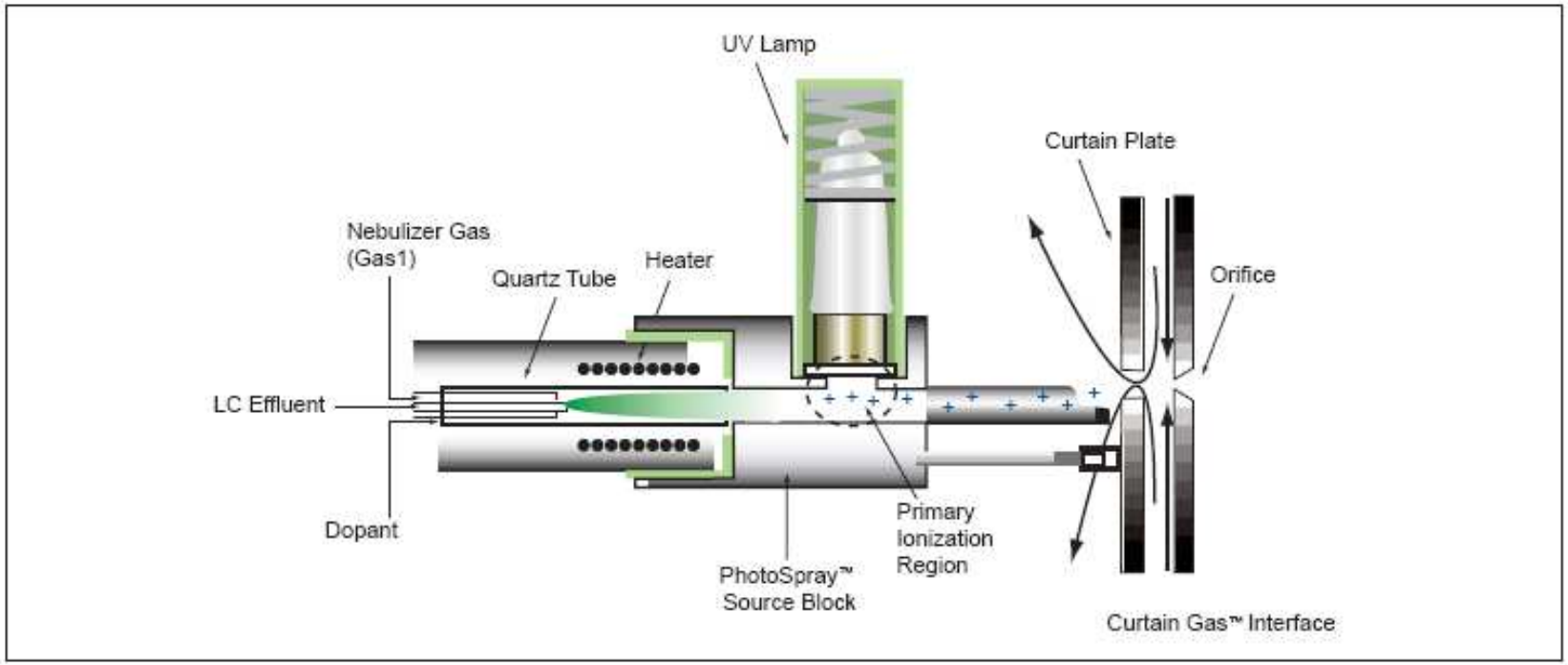
Acetic Acid (< 1%)	Proton Donors
Formic Acid (< 1%)	
Ammonium Hydroxide (< 1%)	Proton Acceptors
Ammonia Solutions (< 1%)	
Trichloroacetic Acid (< 0.1% v/v)	Chromatographic Separation
Trifluoroacetic Acid (< 0.1% v/v)	
Triethylamine (< 0.1% v/v)	Negative Ion Formation
Trimethlyamine (< 0.1% v/v)	
Ammonium Acetate (< 10 mM)	Buffers
Ammonium Formate (< 10mM)	

Atmospheric Pressure Heated Ionisation (APCI)



Solvent molecules (S) being protonated by the corona (SH+), then reacting with the analyte molecule (M) to give the protonated form MH+.

Atmospheric Pressure Photo Ionization (APPI)



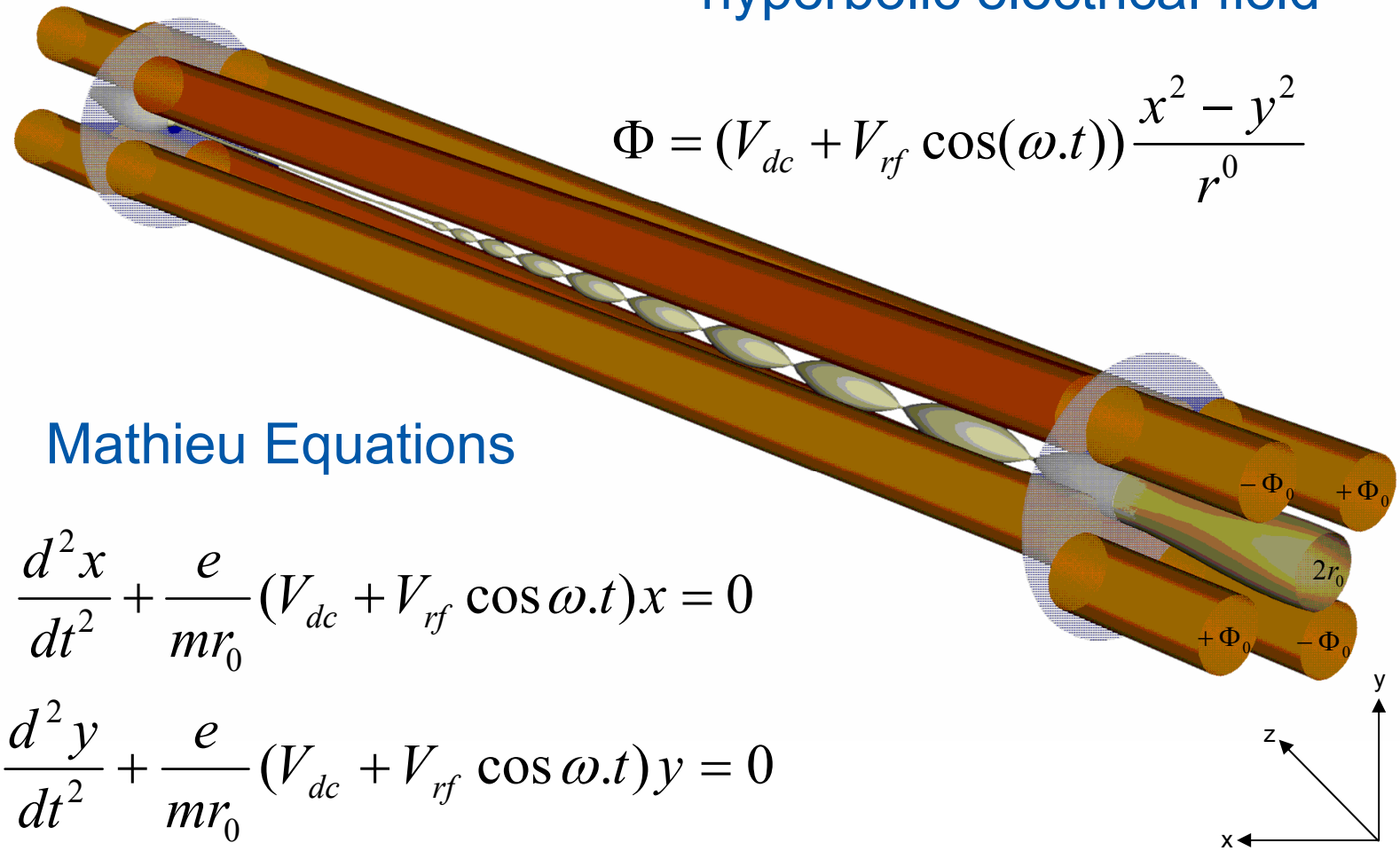
Van-Deemter-Function

$$H_{total} = A + B / v + C * v$$

quadrupol

hyperbolic electrical field

$$\Phi = (V_{dc} + V_{rf} \cos(\omega.t)) \frac{x^2 - y^2}{r^0}$$



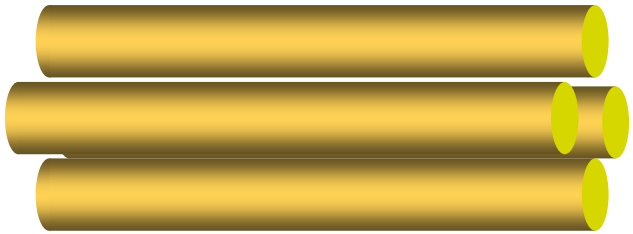
Mathieu Equations

$$\frac{d^2x}{dt^2} + \frac{e}{mr_0}(V_{dc} + V_{rf} \cos \omega.t)x = 0$$

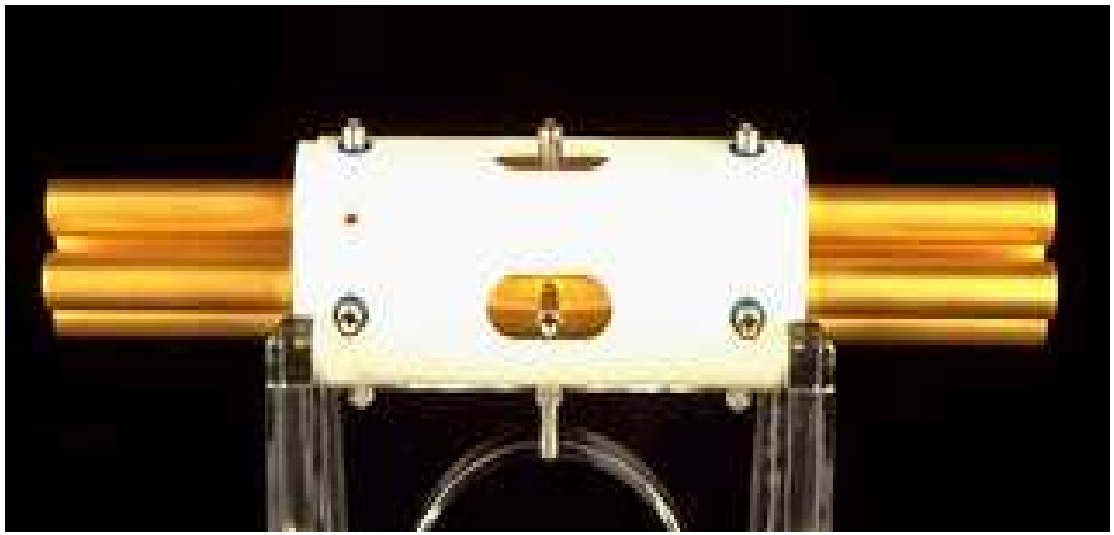
$$\frac{d^2y}{dt^2} + \frac{e}{mr_0}(V_{dc} + V_{rf} \cos \omega.t)y = 0$$

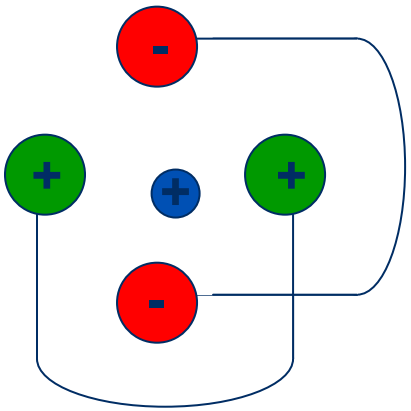
Mass Analyzers

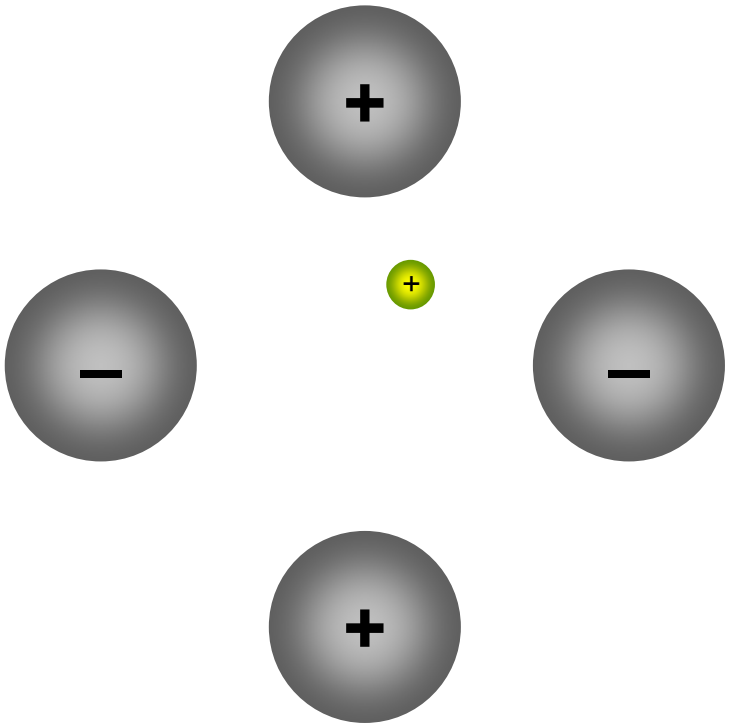
- All mass spectrometers sort ions based on their mass-to-charge ratios (m/z) in a vacuum
- Common analyzer types:
  - Quadrupole
    - single quadrupole
    - triple quadrupole
  - Time of flight
  - Ion trap
  - Hybrid Mass Spectrometers
  - Magnetic and electric sector
  - Fourier transform ion cyclotron resonance



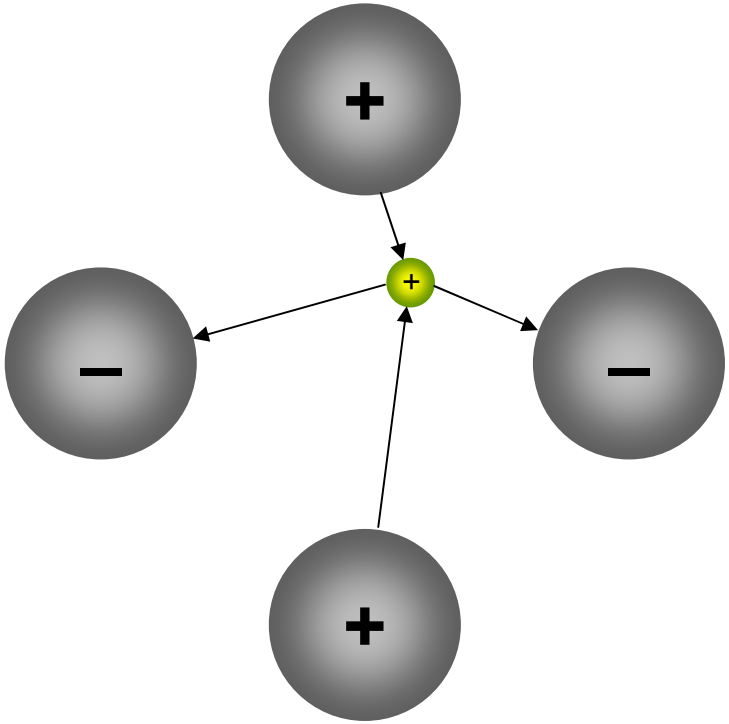
Picture of a Quadrupole



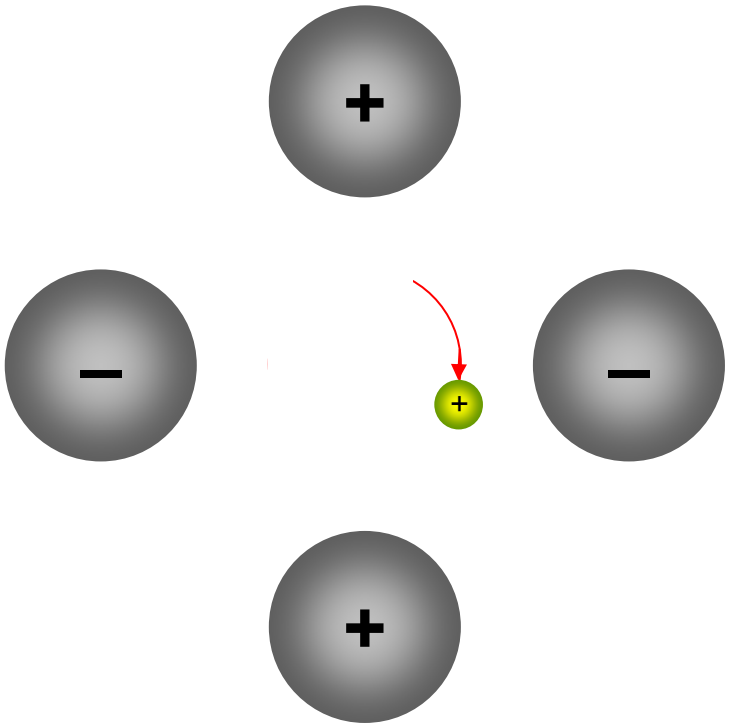




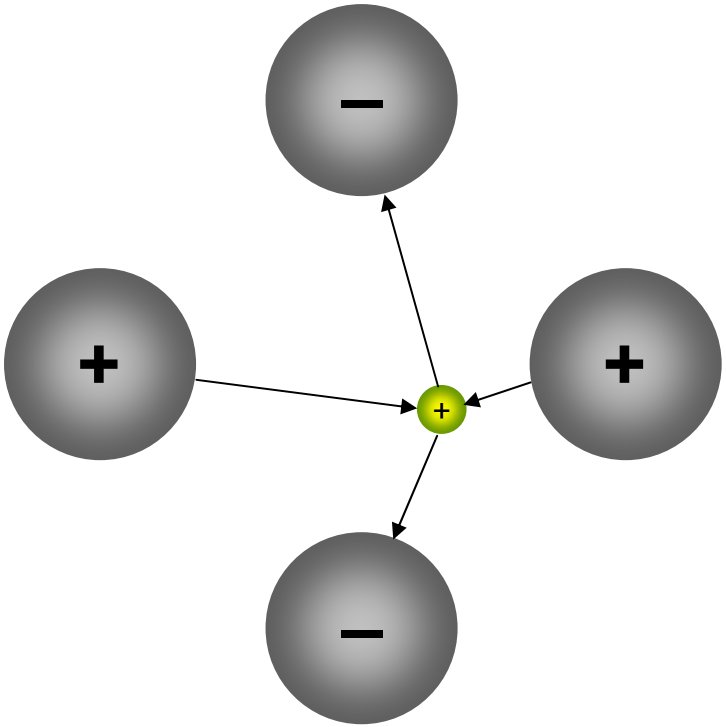
1. Ion enters the quadrupole system



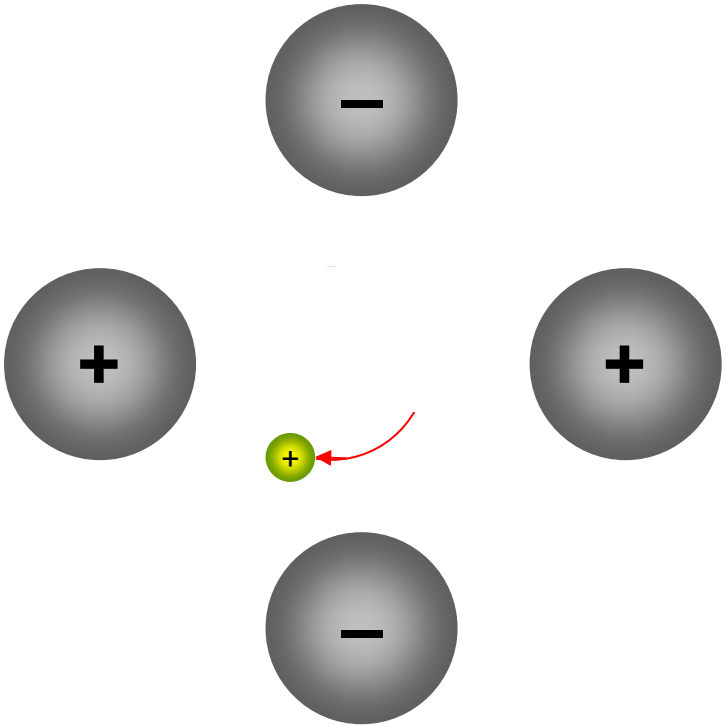
2. Electrical repulsion and attraction, respectively, between quadrupole rods and ion



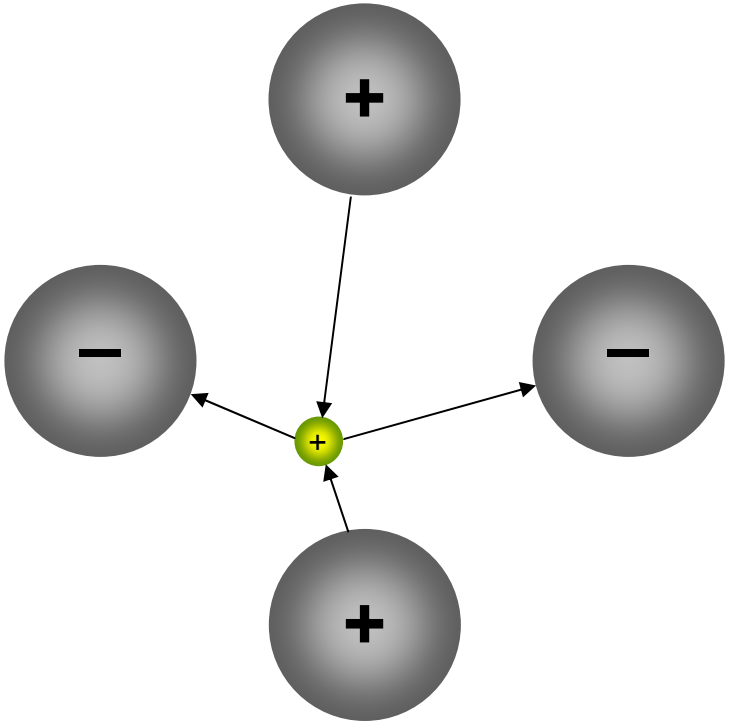
3. Movement of the ion into direction of the nearest quadrupole rod with the opposite charge



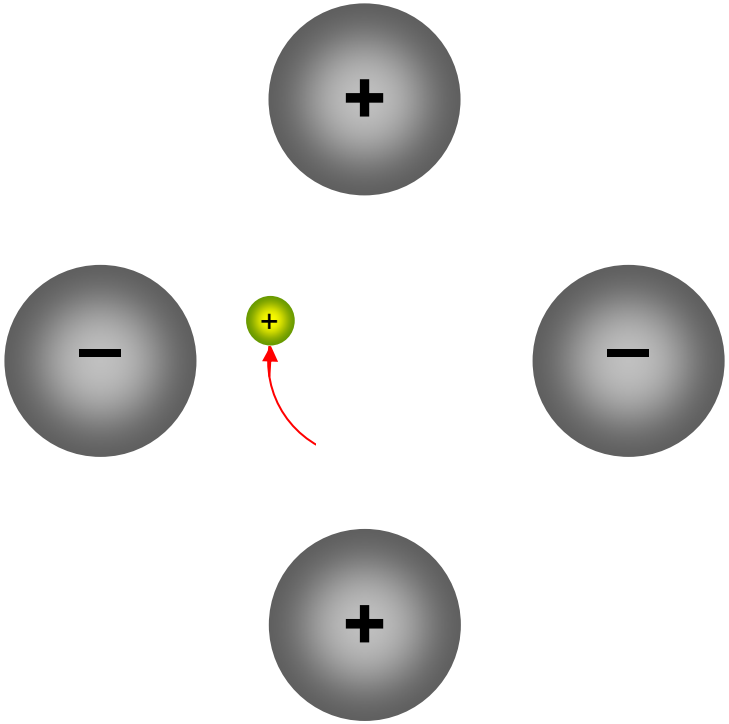
4. RF-voltage changes polarity and electrical repulsion and attraction, respectively, between quadrupole rods and ion



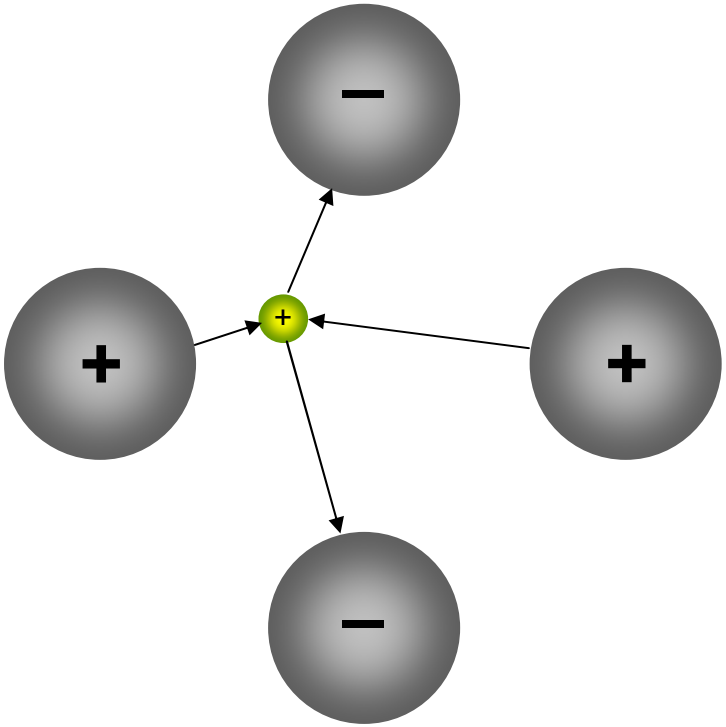
5. Movement of the ion into direction of the nearest quadrupole rod with the opposite charge



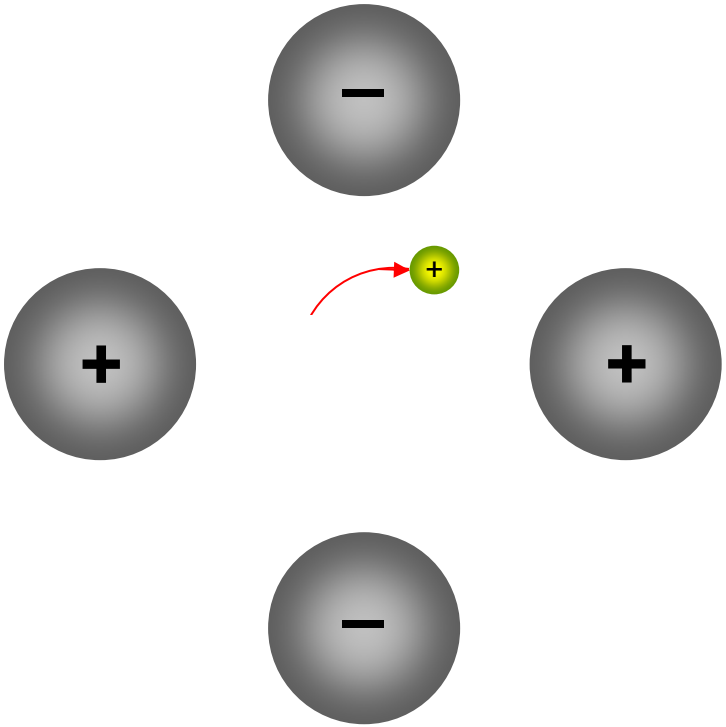
6. RF-voltage changes polarity and electrical repulsion and attraction, respectively, between quadrupole rods and ion



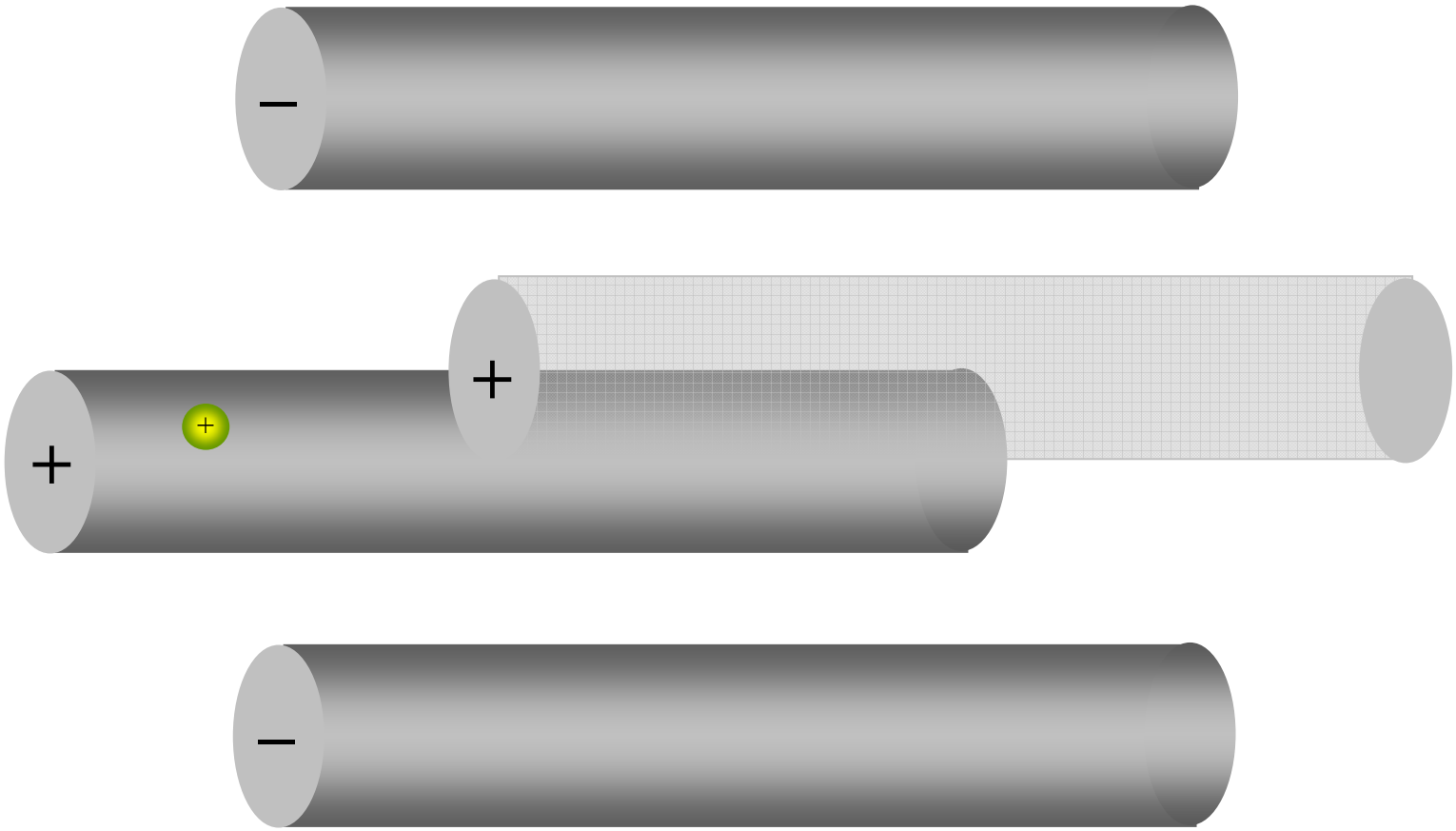
7. Movement of the ion into direction of the nearest quadrupole rod with the opposite charge

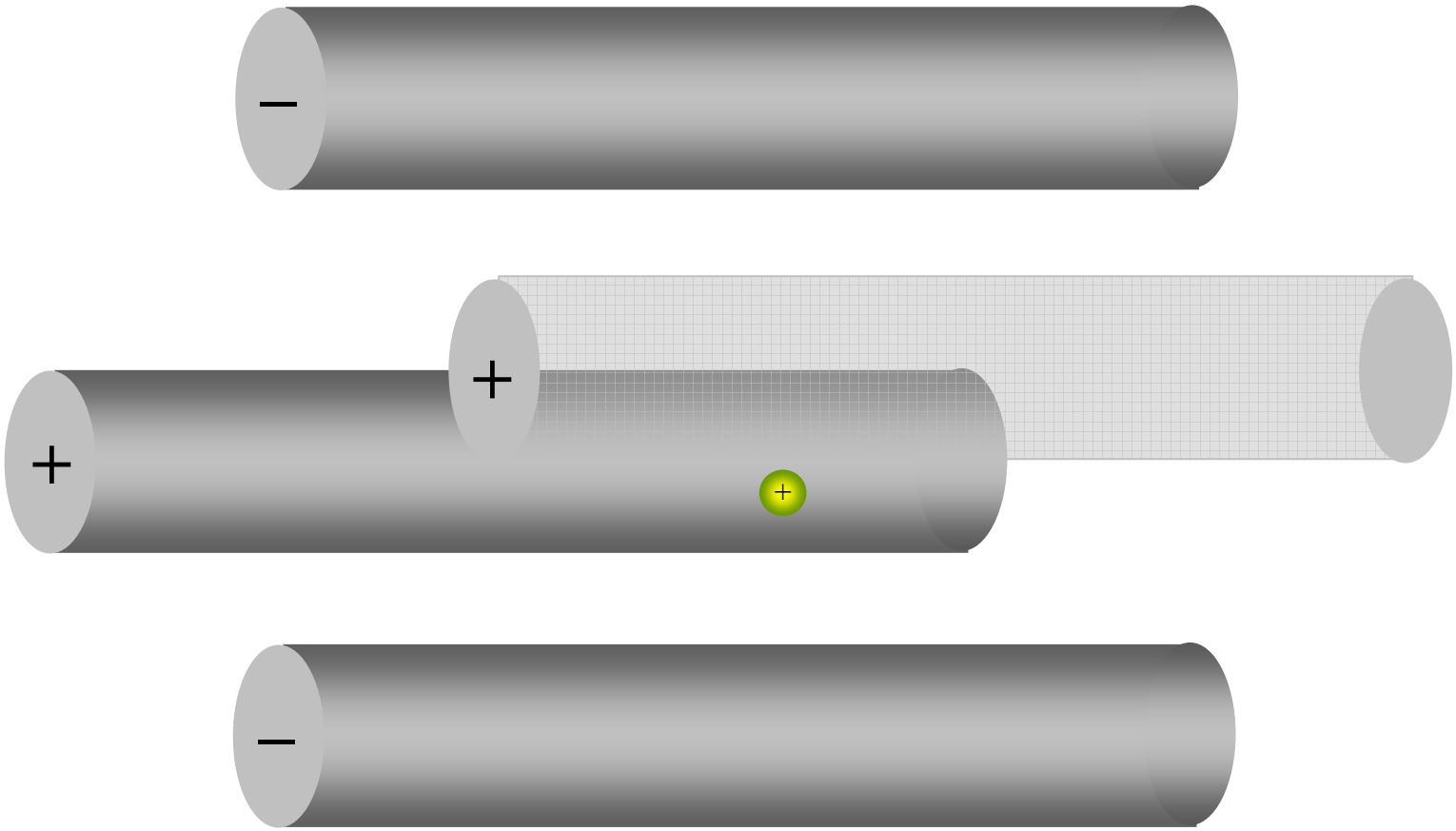


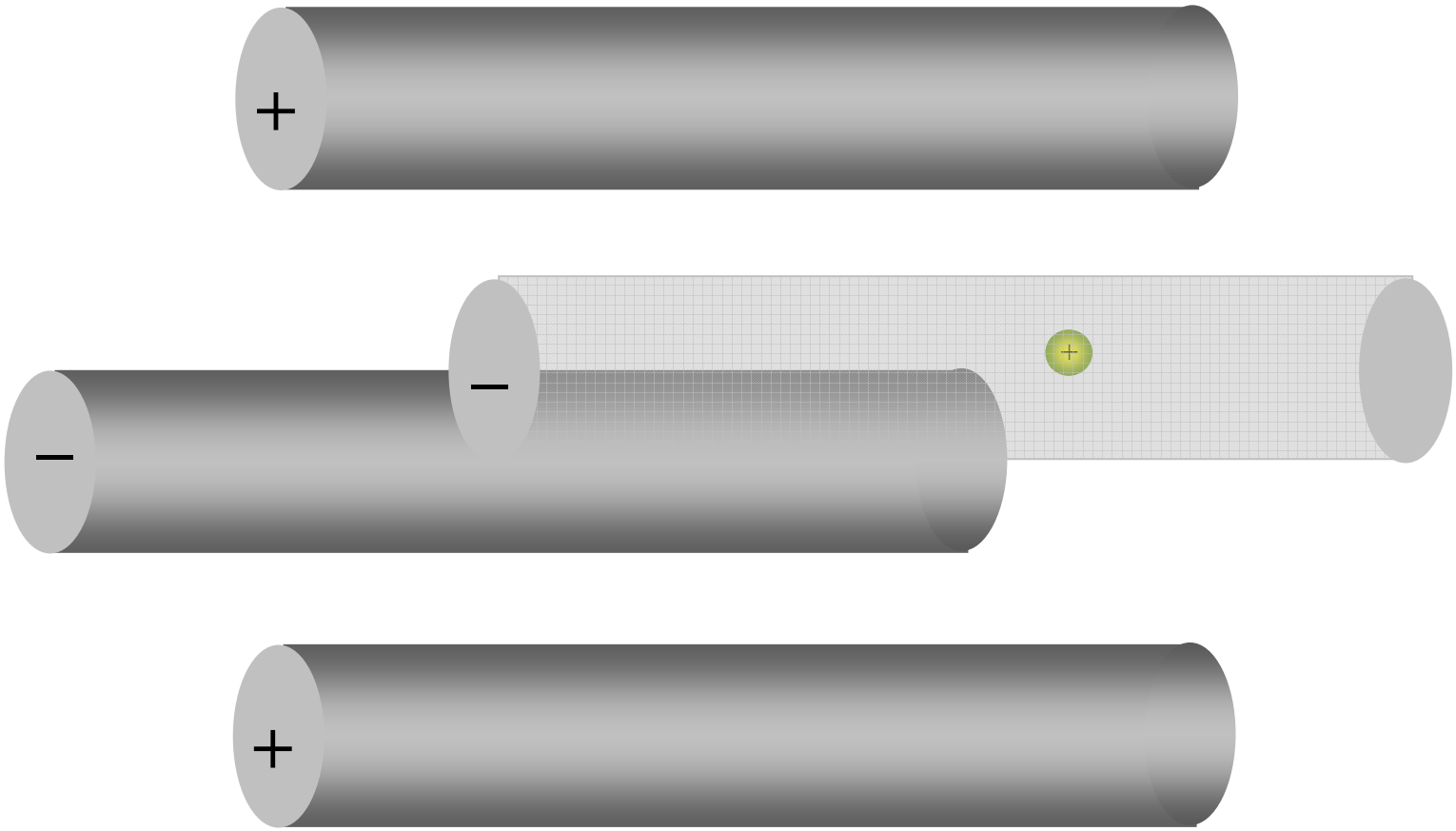
8. RF-voltage changes polarity and electrical repulsion and attraction, respectively, between quadrupole rods and ion

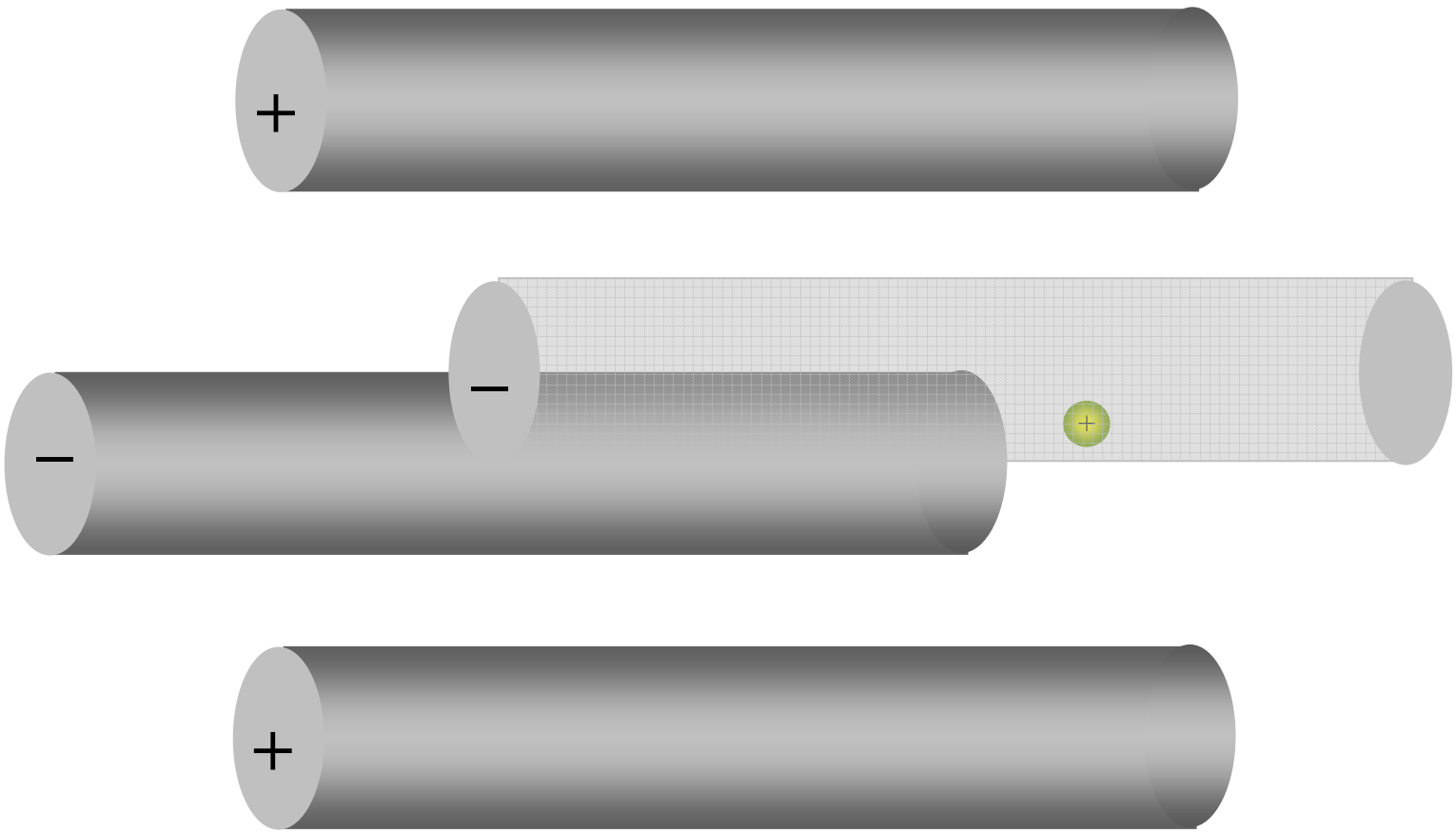


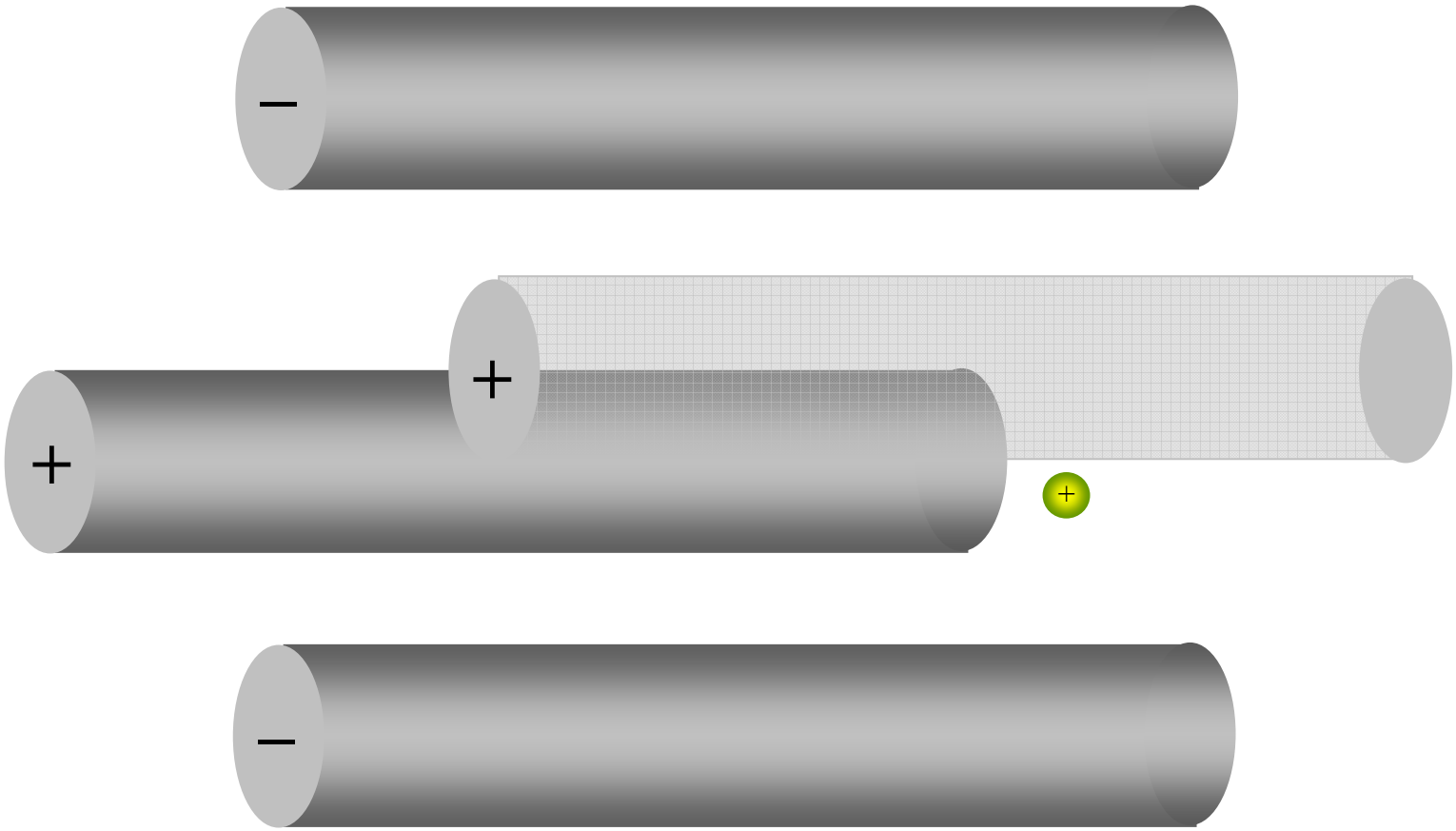
9. Movement of the ion into direction of the nearest quadrupole rod with the opposite charge

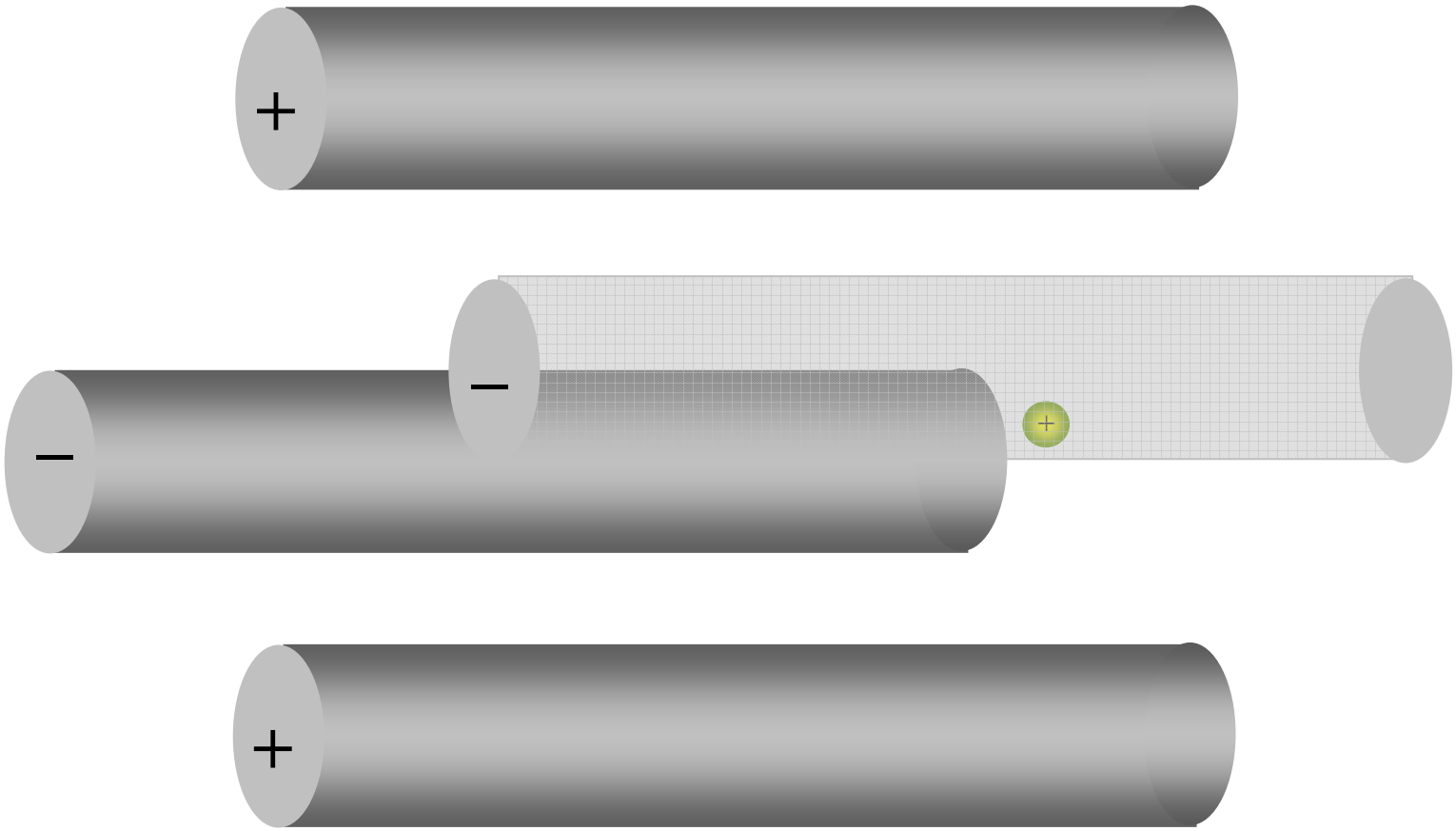




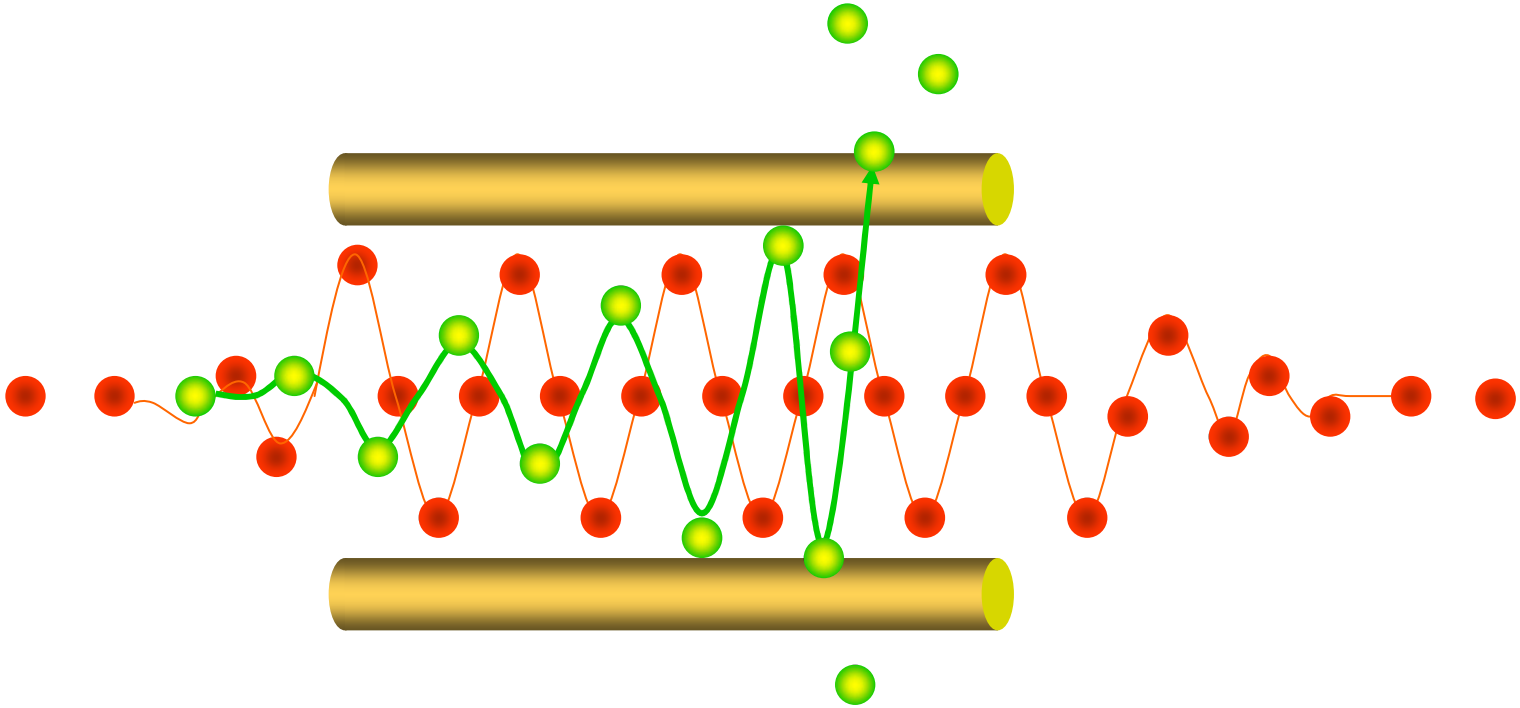






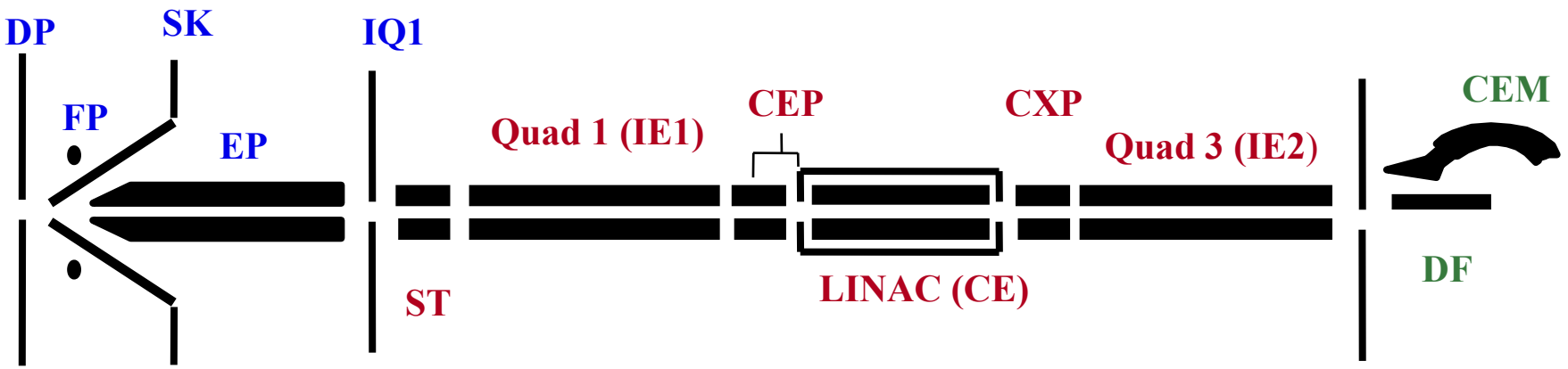


Quadrupole Fundamentals
Stable & Unstable Ion Trajectories



At any RF/DC potential setting only ions of one particular mass/charge ratio have a stable trajectory

Τριπλό Τετράπολο
Lenses and Functions



Interface lens

DP = Declustering potential (orifice plate)
FP = Focusing Potential (focusing ring)
SK = Skimmer
EP = Entrance Potential (Q0 lens)
Iq1 = Interquad lens 1

Lens of the Quadrupole rail

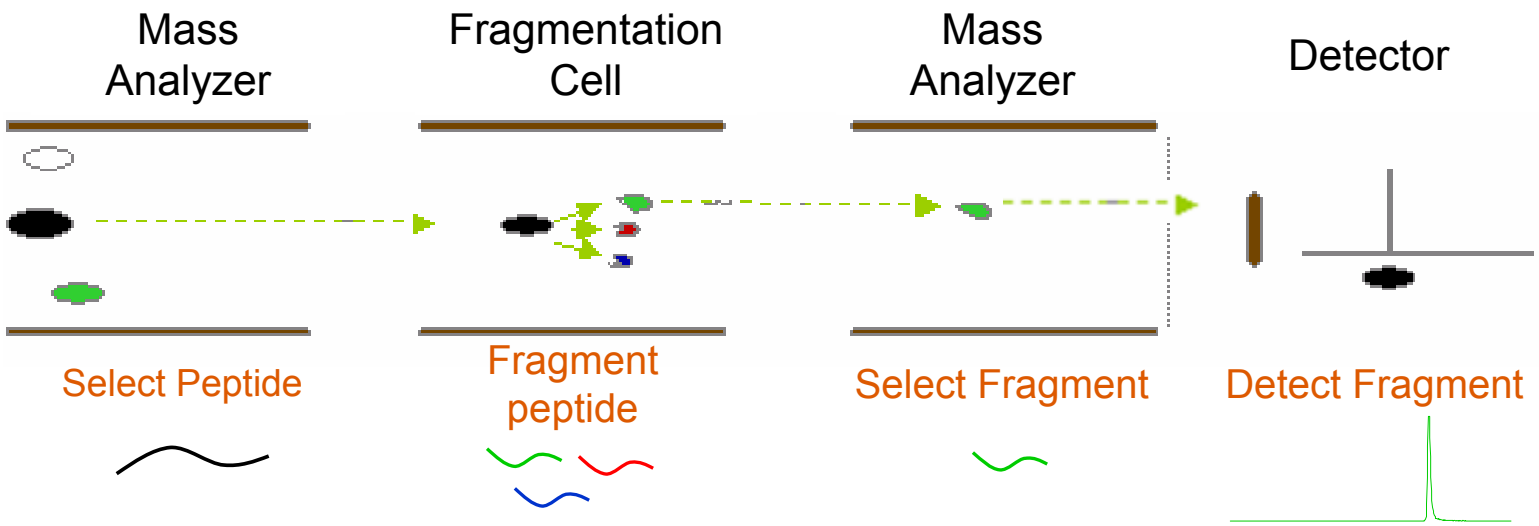
ST = "Stubbies"
Quad 1 = First mass filter (IE1)
CEP = Cell Entrance potential
LINAC = Collision cell (CE), Quad 2
CXP = Cell Exit Potential
Quad 3 = Second mass filter (IE2)

Detector

CEM = Continuous electron multiplier
DF = Deflector

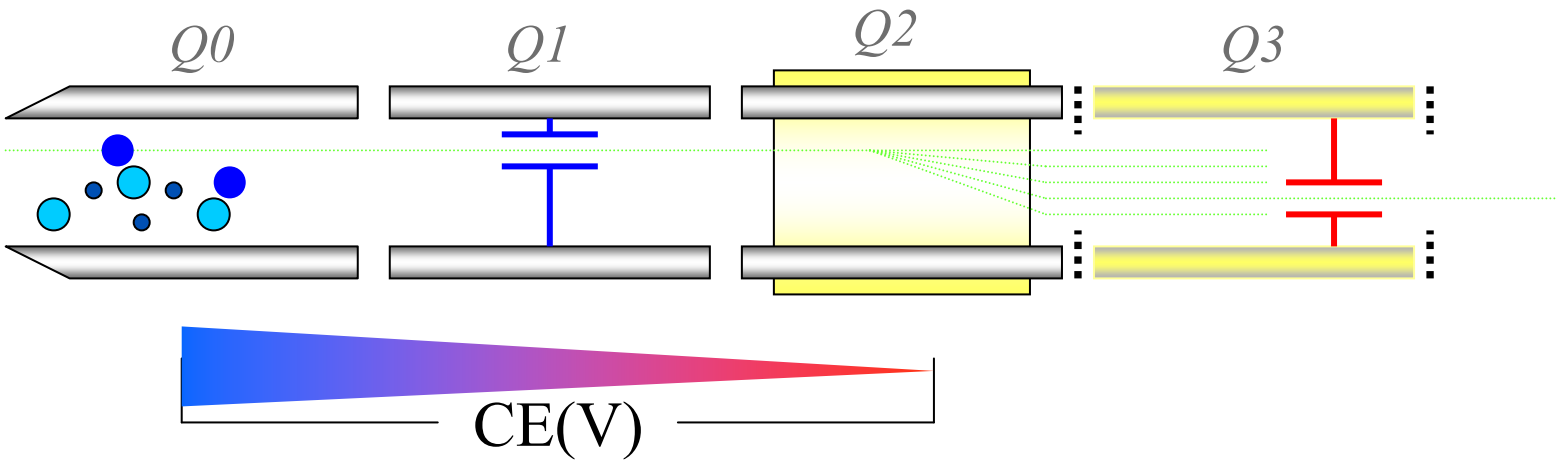
What is MRM?

Multiple Reaction Monitoring



- Highest specificity and sensitivity for detecting components in a complex mixture
- Largest linear dynamic range for quantitation
- Requires triple quadrupole MS capability
- Well accepted as the MS technique for quantitation (Pharmaceutical Industry)

MRM (Multiple Reaction Monitoring)



	Q1	Q3	Dwell time
1	453	254	20ms
2	685	485	20ms
3	453	254	20ms
4	396	274	20ms
5	1098	870	20ms
6	464	222	20ms
7	987	274	20ms
8	887	870	20ms

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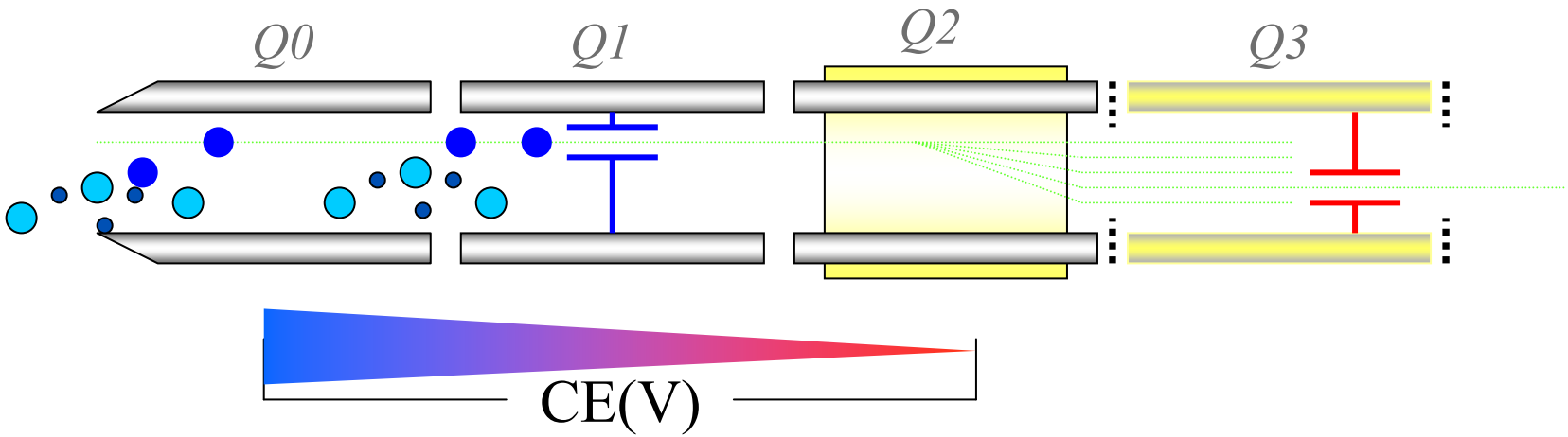

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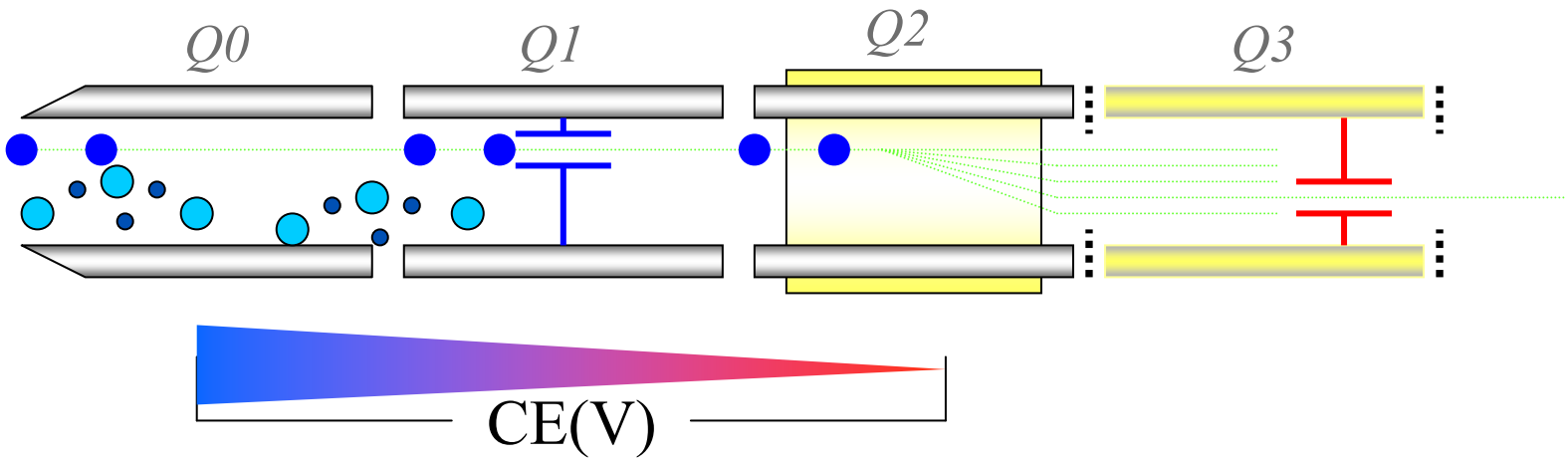

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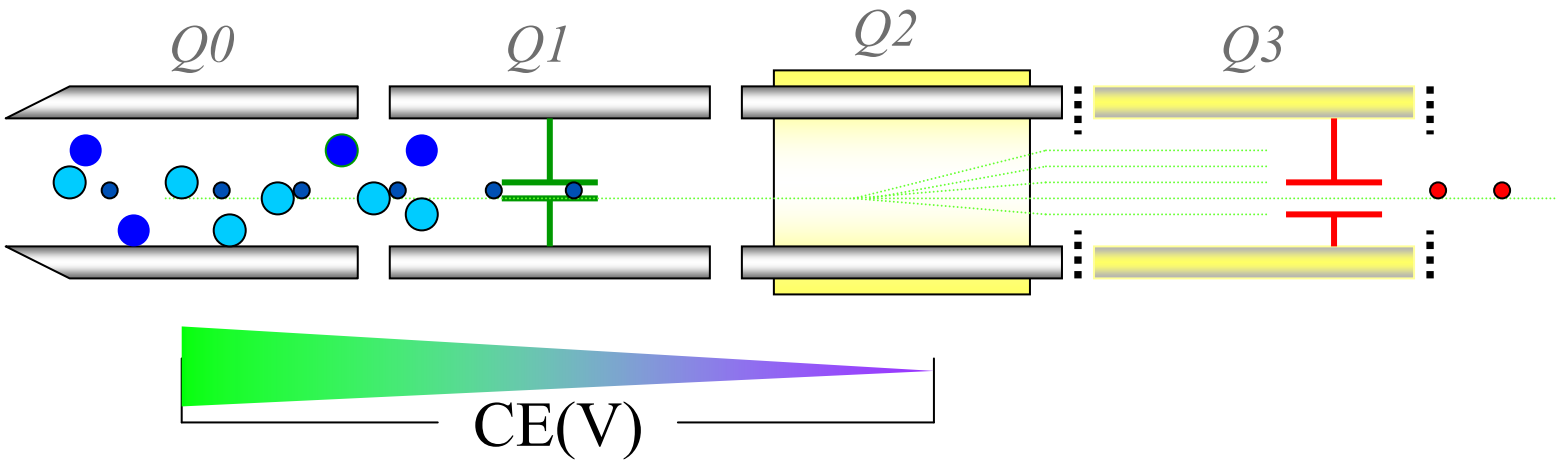

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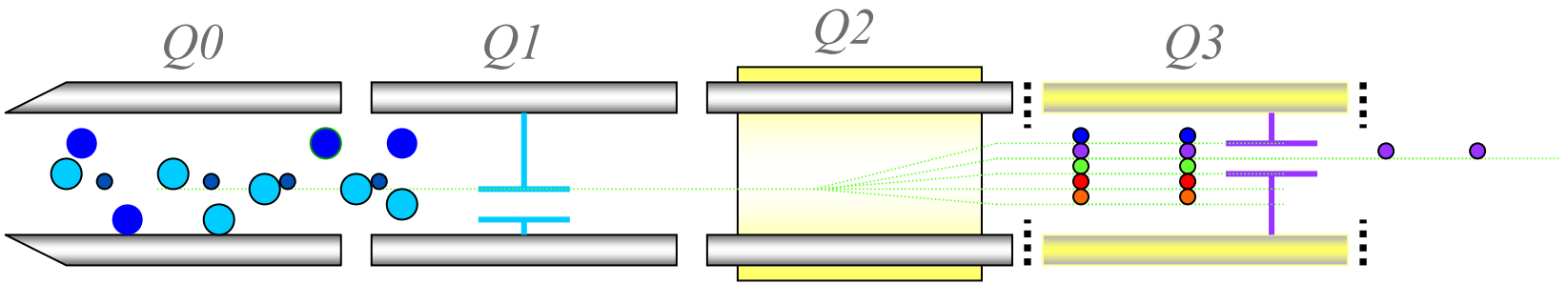

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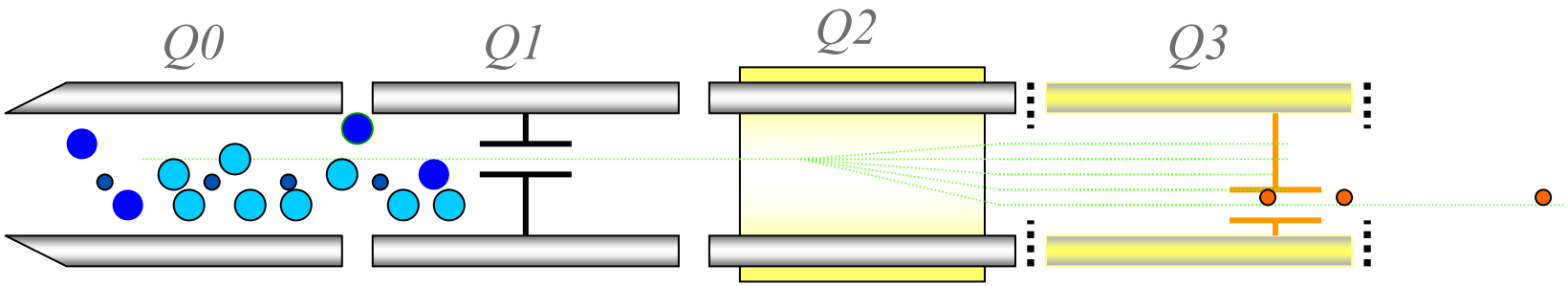

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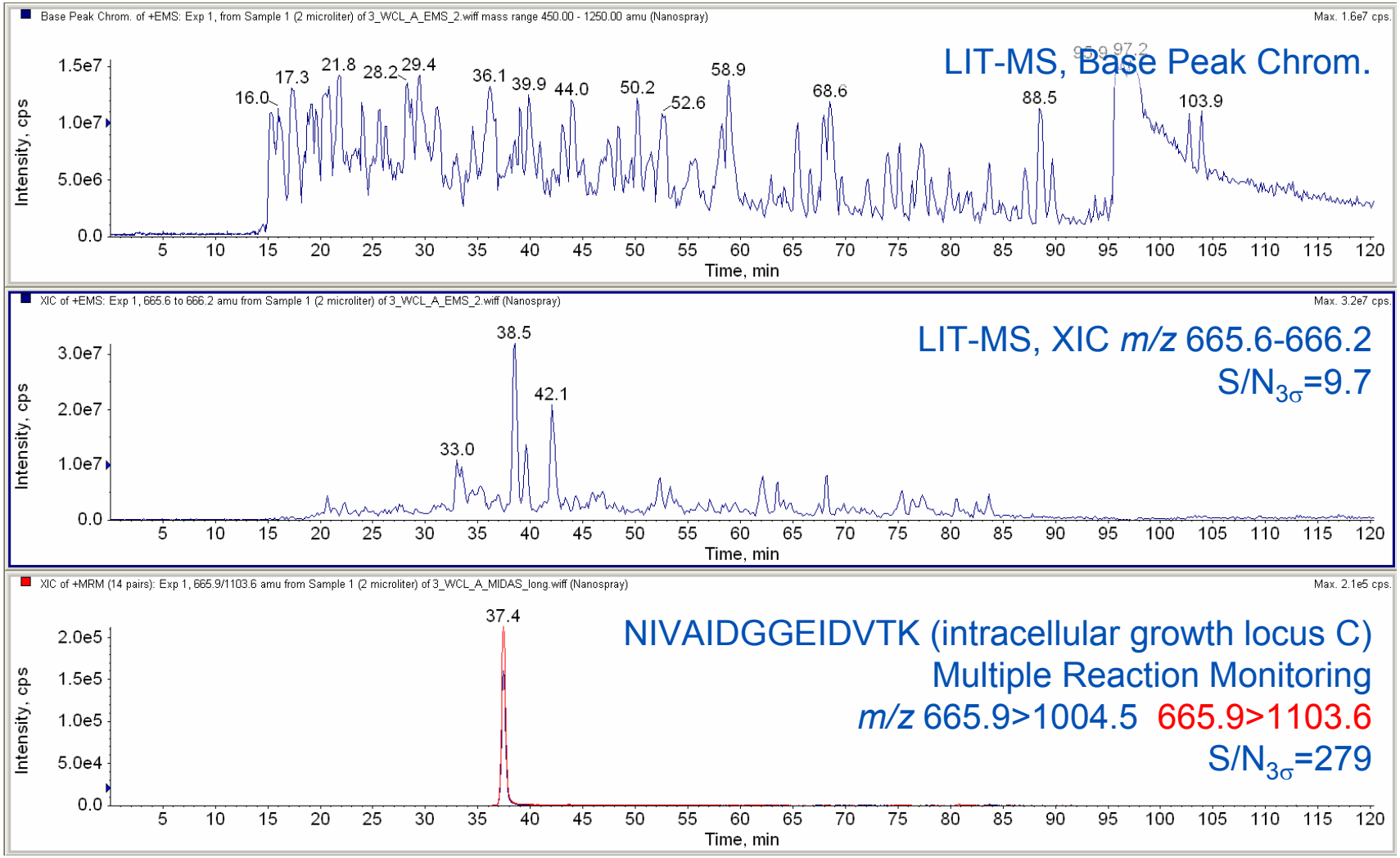
MRM: high selectivity

Francisella tularensis s. SCHU S4, whole cell lysate



Συμπέρασμα

- LC-MS, κύριο εργαλείο ανάλυσης
- Πολλά όργανα αγοράζονται ή αγοράστηκαν τα τελευταία χρόνια στην Ελλάδα
- Ελλειψη εκπαιδευμένου προσωπικού
- Μεγάλες προοπτικές, μεγάλη εφαρμοσιμότητα (βιοεπιστήμες, έρευνα αλλά και ελλεγκτικοί μηχανισμοί)