ROLE OF LC-MS/MS IN MARINE BIOACTIVE COMPOUNDS

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Introduction

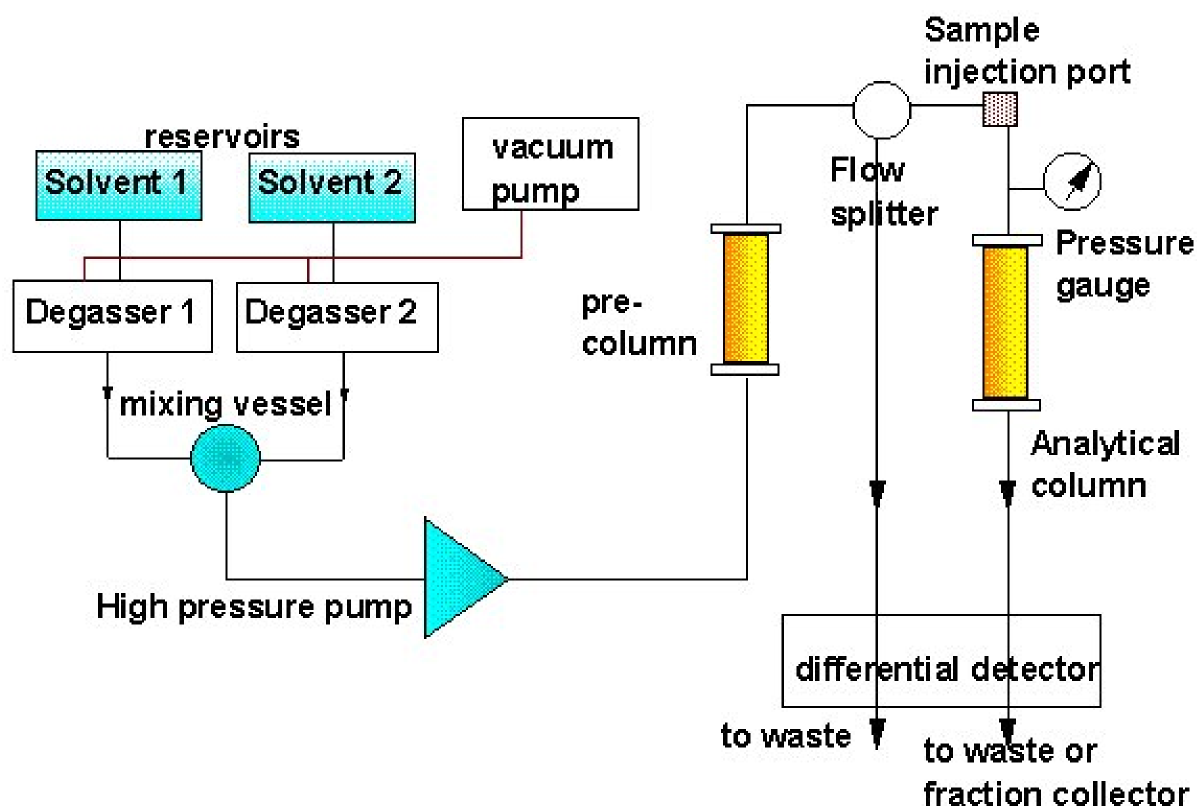
A Mass Spectrometer is an analytical instrument that measures the masses of individual molecules which have been converted into gas-phase ions. Molecules in a liquid-phase need to be converted into a gas-phase for the mass spectrometer to be able to measure them. Ions are separated, detected and measured by their mass-to-charge ratios (m/z). Mass spectrometers hyphenated with liquid chromatograph (LC-MS/MS) are widely used in chemical and biological research now a days, to identify and elucidate structures of unknown metabolites, protein etc. in biological tissue, plant material, microbial broth etc. It is also used for targeted analysis of known compounds such as pesticides, antibiotics, vitamins, amino acids, phospholipids etc. In the field of marine bioactive compounds, LC-MS/MS are widely used to determine metabolite profile of marine plants, fish, crustaceans, micro algae, and microbes. Structure elucidation of marine bioactive peptides is another possible application of LC-MS/MS. It is also used for targeted analysis of phenolic acids, flavonoids, carotenoids, vitamins, phospholipids etc. in the marine plants and animals.

Liquid Chromatography

A high-performance liquid chromatograph (HPLC) or ultra-high performance liquid chromatograph is a common front end of a LC-MS/MS system. HPLC/UHPLC separates mixture of compounds based on the principle of adsorption chromatography where the mobile phase is liquid solvent and the stationary phase is solid sorbent particles tightly packed inside a metal column. When the stationary phase is polar in nature, the type of chromatography is called normal phase chromatography; while in case of reverse phase chromatography the stationary phase is non polar. Reverse phase chromatography is most commonly used with mass spectrometry because of its repeatability, relatively lower maintenance, and chromatographic resolution for wide range of mid-polar to non-polar compounds. Most common type reverse phase stationary phase material is C18, where the silica particle surface is modified with 18 carbon chain length hydrocarbons. Similarly, C30 and C8 columns are used for separation of highly nonpolar and relatively polar compounds respectively. Normal phase

chromatography commonly uses unmodified silica as stationary phase and used for chromatographic separation of polar compound mixture such as fatty acids and tocopherol isomers. In reverse phase chromatography water in combination with acetonitrile or methanol is most common type of mobile phase, where the solvent elution programme starts with high aqueous content and gradually ramped to high organic content. In case of normal phase chromatography, water can not be used as mobile phase because of its interaction with silica particles. A combination of nonpolar and relatively polar solvents is used as mobile phase, where the elution programme starts with high content of nonpolar solvent and the content of polar solvent is gradually increased. The following figure presents different parts of the HPLC/UHPLC.

Figure. Different parts of a liquid chromatograph



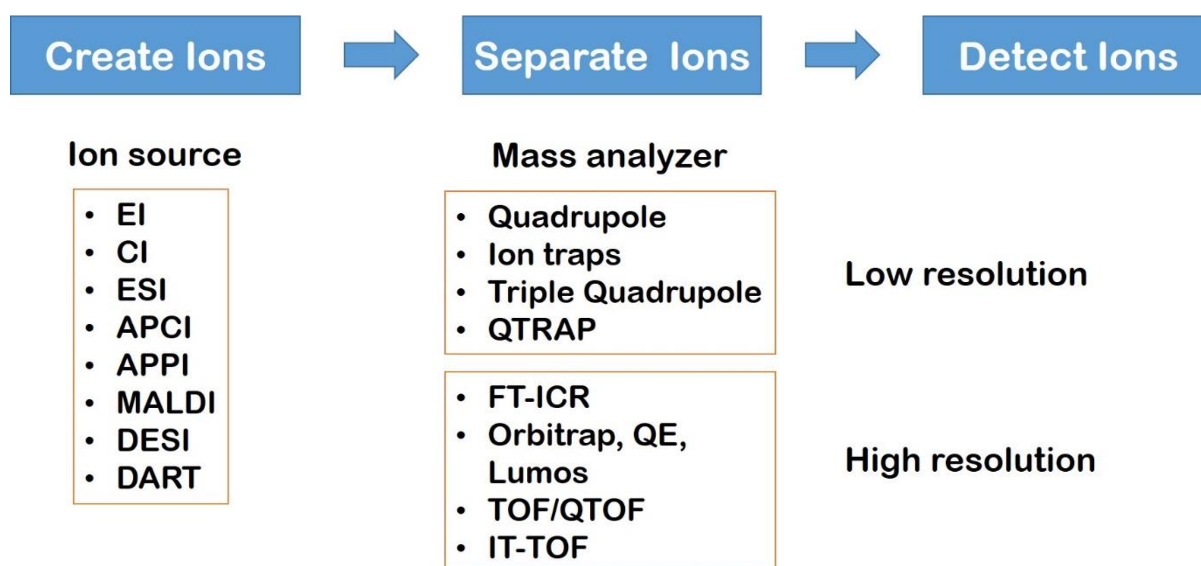
Chromatographic resolution is directly proportional to the length of the chromatographic column, and inversely proportional with the particle size, inner diameter, and pore size. Hence, a short length column with finer particle size, shorter inner diameter, and smaller pore size can achieve the same chromatographic resolution in less time which will take longer in a column of higher length, with bigger particle size, longer inner dia and bigger pore size. However, the solvent back pressure is extremely high in such short columns and can be used only with

UHPLC where the pump is equipped to handle back pressure up to 18000 psi. UHPLC is a popular front end of mass spectrometer due to short analysis time, sharp peak shape, and less consumption of organic solvents.

Mass spectrometer

In a mass spectrometer the compounds introduced in liquid phase form gas phase ions in the ion source. Next the ions are separated in a mass analyzer and finally they reach the detector. The detector shows the output in the data system as a mass spectrum, total ion chromatogram (TIC), base peak ion (BPI) chromatogram, or extracted ion chromatogram (XIC). There are different possible ion sources and mass analyzer combinations in different mass spectrometers which are used for different application needs. The following figure shows a schematic of major parts of a mass spectrometer and lists different possible ion sources and mass analysers.

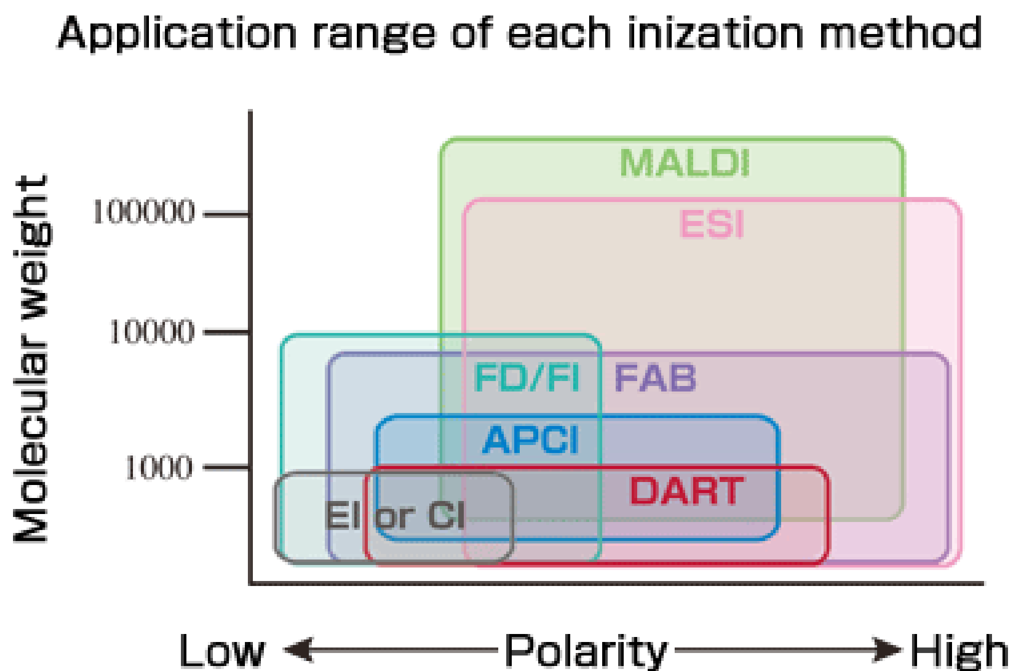
Figure. Schematics of different parts of mass spectrometer



The electron impact (EI) and chemical ionization (CI) ion sources are found in gas chromatograph hyphenated mass spectrometer (GC-MS) and the ionization happens under complete vacuum. EI is a hard ionisation technique, where the molecular weight ion is almost completely broken down into fragments. Hence, for molecular weight determination, CI ion source is preferred in GC-MS; where a pseudo molecular ion with reagent gas (most commonly methane or ammonia) is formed through a soft ionisation technique. Electron spray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo ionisation (APPI), fast atom bombardment (FAB), matrix assisted laser desorption ionisation (MALDI), desorption electron spray ionisation (DESI), direct analysis in real time (DART) are prominent

ion sources in different LC-MS. These ion sources are used based on the polarity and molecular weight range of the target analytes or analyte classes, as shown in the following schematics.

Figure. Application range of different ion sources



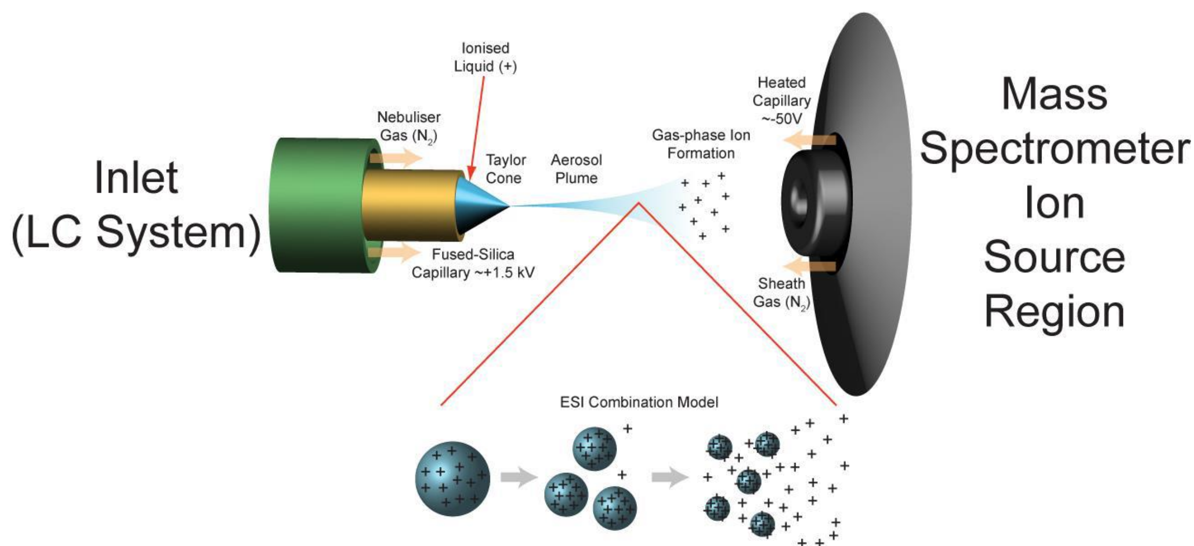
ESI is most commonly used ion source with LC-MS as a wide range of compounds with medium polarity to high polarity, and low to high molecular weight can be analysed. APCI is suitable for compounds with low polarity which do not ionise sufficiently in ESI. APPI is suitable for highly non polar compounds such as persistent organic pollutants. MALDI is prominently used for intact mass determination of proteins.

In ESI ion source, the compound in liquid phase is nebulised through a charged capillary. In positive ionisation mode a positive charge is applied, where in negative ionisation mode a negative charge is applied. The solvent around the droplets containing charged ions is rapidly evaporated by the heater gas and ion source temperature. Hence, the droplets become smaller and smaller, finally releasing only gas phase ions. The ESI is a soft ionisation technique, where the most commonly formed ions are $[M + H]^+$, and $[M - H]^-$, depending on the ionisation operating mode. These ions of a compound are called adduct/pseudo molecular weight ion/parent ion/precursor ion. Some other common adducts found in ESI ion source are listed below.

Positive polarity adduct	Mass difference*	Negative polarity adduct	Mass difference*
$[M + H]^+$	+1.0078	$[M - H]^-$	-1.0078
$[M + NH_4]^+$	+18.0344	$[2M - H]^-$	-
$[M + Na]^+$	+22.9898	$[M - H + H_2O]^-$	+18.0106
$[M + K]^+$	+38.9637	$[M - H + CH_3OH]^-$	+32.0262
$[M + H_2O + H]^+$	+18.0106	$[M - H + CH_3CN]^-$	+41.0265
$[M - H_2O + H]^+$	-17.0027	$[M + Cl]^-$	+36.4609
$[M - 2H_2O + H]^+$	-35.0133	$[M + Br]^-$	+79.9040

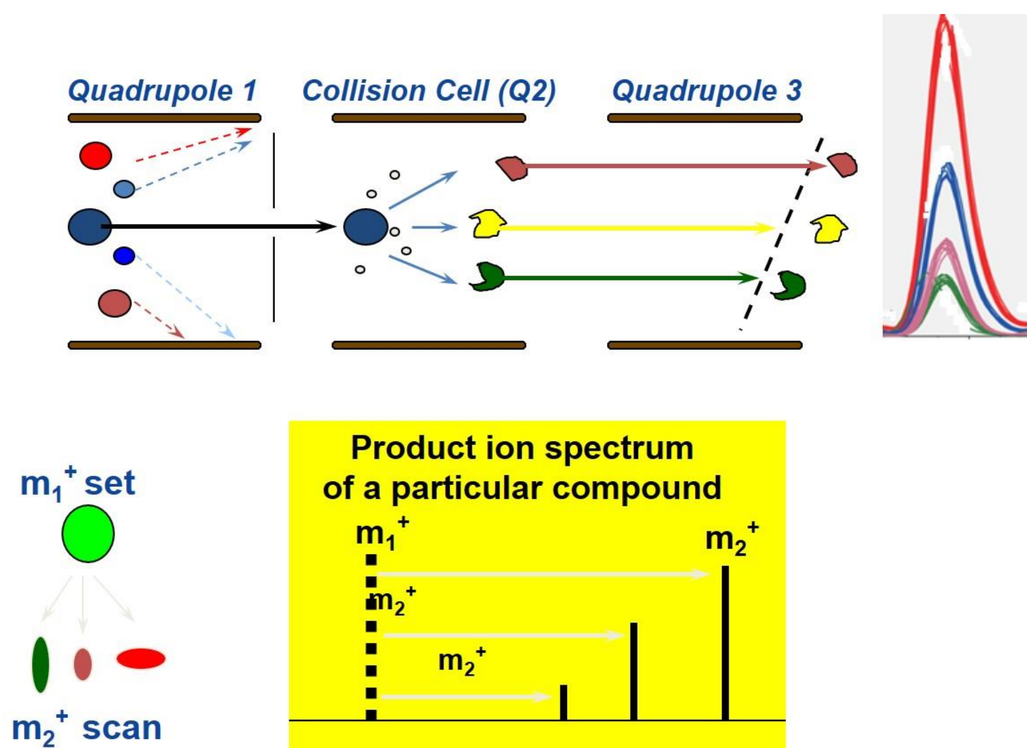
A cone voltage or declustering potential is applied on the ion source cone to further push the generated gas phase ions towards the mass analyser. Hence, the flow rate of nebuliser gas, heater gas, ion source temperature, cone voltage/declustering potential are important parameters that need to be optimised in analyses using ESI ion source. Following is a schematic of the operation of ESI ion source.

Figure. Schematic of ESI ion source.



A quadrupole system uses four cylindrical magnets that are set parallel to each other and function to filter ions based on their mass-to-charge ratio (m/z). The analyzer consists of two pairs of like charged magnets that oppose each other and keep the ions within the ion path of the quadrupole under vacuum. Ions are filtered based on their masses as they traverse the linear ion path. When a linear series of three quadrupoles is used, the resulting triple stage quadrupole analyzer is able to both filter and fragment the ion stream. In most cases, the first (Q1) and third (Q3) quadrupoles act as mass filters, while the second (Q2) quadrupole dissociates ions by having them collide with argon, helium or nitrogen gas. Quadrupole-based mass analysers excel at tracking single ions or reactions for extended periods of time. This is why they are preferentially used in the targeted analysis of compounds, especially known compounds such as drugs and pollutants. This is also why quadrupole mass analysers are often used in the fields of food safety, environmental analysis, clinical and forensic toxicology studies. The triple quadrupole (QQQ) mass spectrometer (MS) consists of a series of three quadrupoles and selects ions of specific mass-to-charge ratios (m/z) when a specific DC/RF voltage combination is applied. The first and third quadrupoles (Q1) act as mass filters, while the Q2 acts as a collision cell. Triple quadrupole MS systems can be operated in a tandem MS/MS assay called Selected

Reaction Monitoring (SRM) (sometimes also called Multiple Reaction Monitoring (MRM)) mode. SRM is a highly selective mode whereby a fixed set of DC and RF voltages is applied to the quadrupole, permitting only one precursor ion, which is measured by its m/z, to pass. After the Q1 filters that specific precursor ion, the Q2 produces product ions via collision of the precursor ion with a neutral gas (e.g., nitrogen) in a process called collision-induced dissociation (CID). Product ions progress to the Q3, where only a specific m/z is permitted to pass. By breaking the ion apart into its component fragments, a given molecular species can be identified not only by its mass but by product identity. In this way, SRM reduces noise and increases selectivity. Following schematic presents the working of a triple quadrupole mass analyser in MRM mode.



LC-MS/MS is a versatile technology with wide range of application in marine bioactive compound analysis. LC-MS/MS can be used for free amino acid analysis in serum, tissue or plant material extracts. The instrument with ESI and MALDI ion source has prominent application in the field of proteomics and peptide sequencing of bioactive peptides. The technique is also used for structural elucidation of bioactive compounds through molecular weight determination, and tandem mass spectra fragmentation pattern. High resolution mass spectrometer can be used for high throughput metabolomic profile of biological materials and can derive important insights in biological experiments.