



# High pressure liquid chromatography (HPLC)

- separation of complex mixtures
- positive and negative ions
- proteins, peptides and DNA

High pressure liquid chromatography (HPLC) enables the dissection of complex mixtures into individual constituents. Mass spectrometry (MS) is an analytical technique that allows sensitive and accurate determination of the molecular weight and additional characterization of ionised compounds in the gas phase.

The combination of HPLC and MS combines the power of both techniques, allowing the dissection and characterization of complex mixtures of a variety of compounds in a single analysis.

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**PHILIPS**

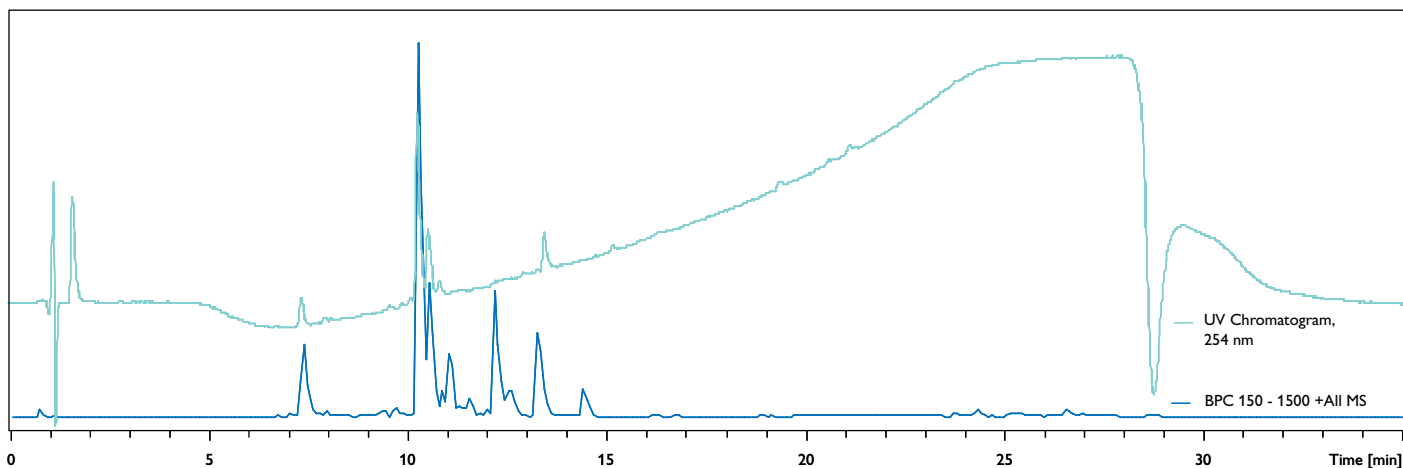


Fig. 1: UV chromatogram (light blue) and MS positive ion chromatogram (dark blue) of the same HPLC separation of a crude mixture obtained after organic synthesis. The MS signal provides a different view of the sample compared to the UV signal.

### The principle of HPLC

High pressure liquid chromatography (HPLC) enables the dissection of complex mixtures into their individual components. This can be achieved by making use of different interactions of compounds in solution, with a stationary phase. By selecting a particular combination of a mobile and stationary phase, the mode of separation can be chosen and optimised.

A commonly used form of HPLC is so-called *reversed phase* HPLC. Here, a hydrophobic stationary phase is used and compounds are loaded under aqueous conditions. Consequently, hydrophobic compounds preferably interact with the stationary phase, rather than remaining dissolved in the aqueous phase. After loading, the conditions in the liquid phase are slowly changed from aqueous to organic. This results in the elution of compounds from the stationary phase in order of hydrophobicity. Detection of eluting compounds yields an output such as shown in Figure 1, called a chromatogram. If needed, quantification of compounds can be performed by calculating the area under the curve of the peaks in the chromatogram. Depending on the types of stationary phase, the interaction strength in the reversed phase can be varied. Next to reversed phase HPLC, several other types of columns are available differing in interaction type and/or

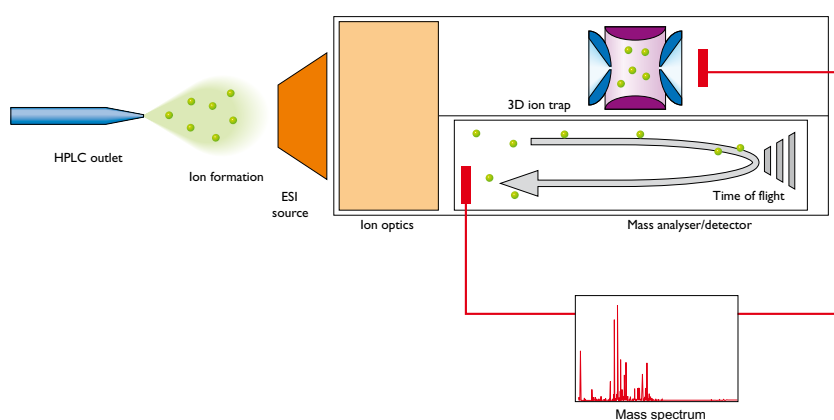


Fig. 2: Schematic setup of a mass spectrometer: the outlet of the LC is coupled directly to the electrospray ionisation source. Ions formed are introduced into the mass spectrometer and ion optics are subsequently used to focus the ions. The mass analyser, which can for example be a 3D ion trap or Time of Flight, separates by mass the ions that finally reach the detector yielding a mass spectrum.

strength. Some examples are given in Table 1. Moreover, chromatographic modes can be used in sequence, enabling the separation of complex mixtures into individual compounds based on a variety of (orthogonal) characteristics. Finally, depending on sample availability, the chromatographic scale can be varied (see 'Characteristics').

### Detection

Even though UV is a sensitive detection method, not every compound exhibits UV absorption. This can cause problems in the detection and quantification of particular analytes. Therefore, a mass spectrometer can be used as (a second) detector. In this way, the range of detectable compounds can be improved (see Figure 1).

### The principle of mass spectrometry

A mass spectrometer generally consists of three modules: an ion source, a mass analyser and a detector. This is depicted in Figure 2. When using an electrospray ion (ESI) source, compounds in an acidified liquid phase are introduced into the mass spectrometer through a heated capillary. Consequently, liquid

Table 1: Examples of some column types available for a wide range of separations.

Type of column	Mode of separation
Reversed / normal phase	Differences in hydrophobicity (polarity)
Ion exchange	Differences in charge at a certain pH
Gel filtration	Differences in size (molecular weight)
(Bio-)affinity	Differences in interactions with a ligand

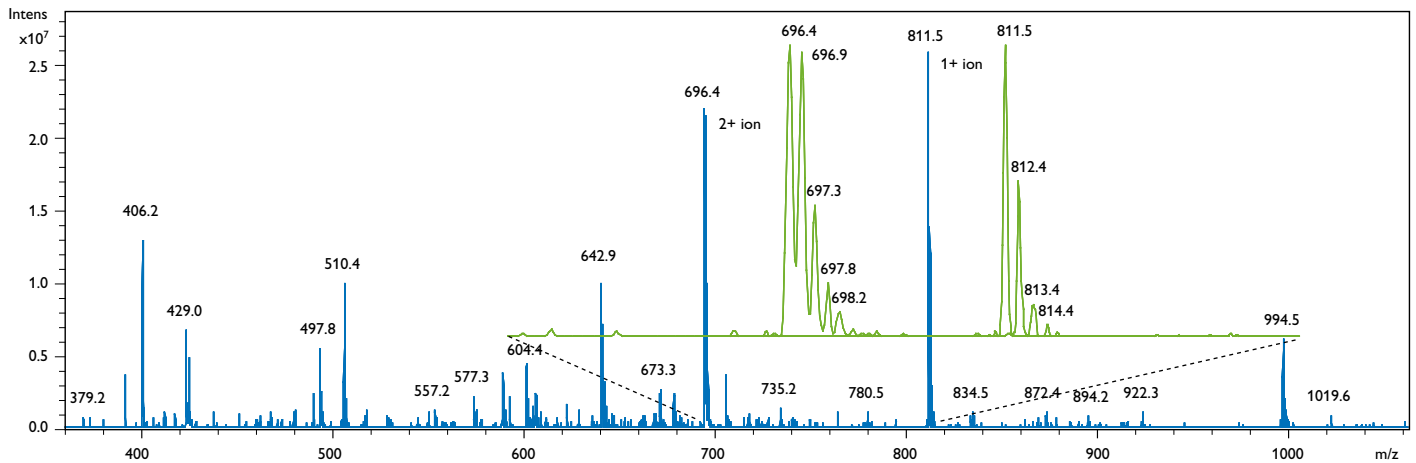


Fig. 3: Typical mass spectrum, containing a large number of ions. In the inset an example of both a singly and doubly charged ion are given.

vaporizes and ions are formed. The resulting ions enter the mass analyser, which can, for example, be an ion trap (IT) or a time of flight (ToF) tube. The mass analyser separates ions based on their mass and finally a detector (the red squares in Figure 2) is used for detection of the ions. This can be done either in the positive or the negative ion mode.

### The mass spectrum

A mass spectrometer determines the mass-over-charge ( $m/z$ ) ratio of ions, which can be used to calculate the mass of the corresponding molecules.

Due to the occurrence of natural (stable) isotopes, the vast majority of compounds is represented by multiple peaks in the mass spectrum. Figure 3 shows a typical mass spectrum, in which several ions of 'average organic compounds' are present. Some of these ions are singly charged, others are doubly (or multiply) charged as can be seen in the inset. The difference between such charge states can be determined by the distance between the isotope peaks.

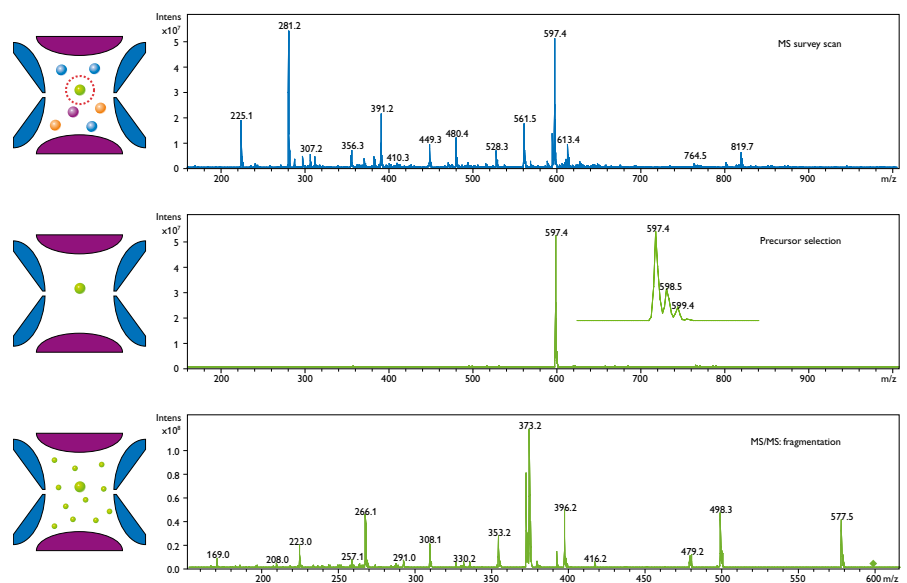


Fig. 4: The process of MS/MS fragmentation in a (schematically drawn) 3D ion trap, illustrated for an ion with an  $m/z$  of 597.4. After detection of the ion in an MS survey scan (top panel), the ion of interest ('precursor') is selected (middle panel) and subsequently fragmented (bottom panel), yielding information about the composition of the ionised molecule.

### Ion trap mass spectrometry: compound fragmentation

An ion trap mass spectrometer cannot only determine the  $m/z$  ratio, but is also capable of performing so-called *tandem mass spectrometry* (MS/MS or  $MS^2$ ), in which a certain ion is selected for fragmentation. This can help to identify particular elements in a molecule. An example of a fragmentation study is depicted in Figure 4. During fragmentation, (labile) bonds in the molecule break, thereby producing fragment ions that are characteristic for certain chemical moieties. In some cases, highly labile bonds are present, and fragmentation will yield only little compositional information. In that

case it is possible to perform sequential fragmentation ( $MS^n$ ), which enables isolation and subsequent fragmentation of fragment ions. This, in theory, makes it possible to break a molecule down to very small pieces, yielding more detailed information on the molecular structure.

### Time of flight mass spectrometry: high mass accuracy

In contrast to an ion trap, a time of flight (ToF) is not capable of performing tandem mass spectrometry. On the other hand, it

can determine molecular masses of ionised compounds with much higher accuracy (< 5 ppm) than the ion trap. Accurate mass determination can also aid in resolving the elemental composition of an unknown compound. Moreover, determining a relative mass shift on a particular compound resulting from a chemical modification can help in determining the reaction efficiency. In Figure 5 a magnified version of a mass spectrum generated by a ToF is given, which shows baseline resolution for individual isotopes of a singly charged ion.

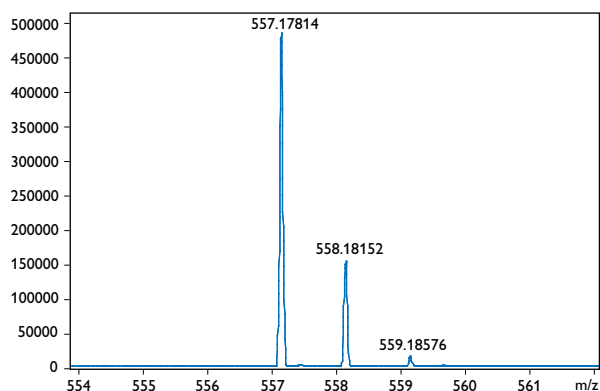


Fig. 5: Example of an accurate mass determination, using an ESI-ToF mass spectrometer.

### Applications

- Characterization of reaction mixtures resulting from organic synthesis
  - purity of the starting material
  - the presence of remaining starting compounds and/or unexpected side products
  - identification based on fragmentation patterns
- Quantitation of (a) specific compound(s) within a mixture
- Identification and characterization of biomolecules
  - protein sequencing
  - molecular weight determination of proteins or DNA

### A typical application: Characterization of chemical modifications on proteins

Antibodies are proteins that are increasingly used for diagnostic purposes. To visualize them in cells or the human body, fluorescent or radioactive reporter groups have to be attached to the antibody. The resulting modified antibodies can be studied using LC-MS, giving insight into the labelling efficiency. By measuring the intact protein both before and after labelling, the (average) number of modifications present per protein molecule can be determined using the mass difference before and after labelling.

Subsequent proteolytic digestion of antibodies into peptides and LC-MS/MS analysis of the resulting peptide mixtures allows for comparison of unmodified and chemically modified proteins. This enables the localization of chemical modifications in the primary sequence of the protein. Large numbers of protein 3D structures are given in public databases. This information can be used to determine the site of modification on a protein. In this way, information is obtained about possible activity loss as a result of the chemical modification of amino acids that are essential for protein function.

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### Characteristics

#### Information obtained

- Sample composition, molecular weight and structural information on individual components
- Qualitative and (semi-)quantitative information

#### Sample type

Solid or liquid (dissolved in aqueous buffers or solvents like DMF or DCM)

#### Analytical range

- Percentages using 'normal' HPLC dimensions (2.1x100mm columns, 0.3 mL/min flow rates)
- Femtomoles using 'capillary' HPLC (0.3x200mm columns, 1 µL/min flow rates)
- 'Nano' LC lowers column dimensions and flow rates even further, thereby increasing sensitivity

#### Typical sample size

0.5-10 µL

#### Accuracy

< 5ppm (for LC-ESI-ToF analyses)

#### Destructive

Yes

