# The use of liquid chromatography/mass spectrometry (LC/MS) in biological monitoring

## Abbreviations

| | |
|---|---|
| ADS | Alkyl-diol-silica phase |
| AGP | $\alpha_1$-acid glycoprotein |
| APCI | Atmospheric pressure chemical ionisation |
| API | Atmospheric pressure ionisation |
| APPI | Atmospheric pressure photo-ionisation |
| CE | Capillary electrophoresis |
| CEC | Capillary electrochromatography |
| CGE | Capillary gel electrophoresis |
| CIEF | Capillary isoelectric focusing |
| CNL | Constant neutral loss |
| CRM | Charge residue model |
| CZE | Capillary zone electrophoresis |
| DEHP | Di(2-ethylhexyl)phthalate |
| EPI | Enhanced product ion |
| ESI | Electrospray ionisation |
| FT-MS | Fourier transform mass spectrometer |
| GC/MS | Gas chromatography/mass spectrometry |
| HLB | Hydrophilic lipophilic balance |
| HPLC | High performance liquid chromatography |
| ICR-MS | Ion cyclotron resonance mass spectrometer |
| IEM | Ion evaporation model |
| ISRP | Internal surface reversed phase |
| ITP | Isotachophoresis |
| LC/MS | Liquid chromatography/mass spectrometry |
| LPS | Large particle support |
| MEKC | Micellar electrokinetic (capillary) chromatography |
| MRM | Multiple reaction monitoring |
| m/z | Mass-to-charge ratio |
| PAH | Polycyclic aromatic hydrocarbons |
| pCEC | Pseudo-capillary electrochromatography |

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| | |
|---|---|
| QqTOF | Hybrid quadrupole time-of-flight mass spectrometer |
| RAM | Restricted access media |
| RPC | Reverse phase chromatography |
| SIM | Selected ion monitoring |
| S/N | Signal-to-background noise ratio |
| SPE | Solid phase extraction |
| SPS | Semi-permeable surface |
| SRM | Selected reaction monitoring |
| TFC | Turbulent flow chromatography |
| TOF-MS | Time-of-flight mass spectrometer |
| UV | Ultraviolet |

## 1 Introduction

Liquid chromatography/mass spectrometry (LC/MS) is a new and unique way of coupling the well-known analytical techniques of liquid chromatography and mass spectrometry. Starting with the pioneering research of Dole et al. [1] and Tal'roze et al. [2–4] at the end of the 1960s, this new technique achieved its greatest international scientific recognition to date with the award of the Nobel Prize for Chemistry to John B. Fenn in 2002.

Whereas gas chromatography/mass spectrometry (GC/MS) is one of the established routine techniques that is widely used for many different fields of application in analytical laboratories on account of the low purchasing price of the instruments, LC/MS is still at the stage of being introduced to the majority of potential users. LC/MS has sparked keen interest since it has proved successful in the determination of many organic compounds in the last twenty years, in particular polar, thermally labile substances and those with high molecular weights (>1000 u) that are difficult or impossible to analyse by means of GC/MS. It is estimated that approximately 80% of all the known organic compounds can be analysed using LC/MS, whereas only about 20% of all known organic species are accessible to GC/MS analysis without previous derivatisation [5]. In the last five years more than a dozen new LC/MS systems have become commercially available at reasonable prices. This has acted as an additional incentive to interested scientists to use LC/MS. It should be noted that basic research in this field has by no means been concluded. In addition to the steadily growing use of LC/MS systems for routine analysis, new instruments are still being developed and optimised for innovative applications.

When used for the purpose of biological monitoring (BM), LC/MS offers new opportunities in the fields of occupational and environmental medicine, especially for the specific and highly sensitive assay of polar metabolites of health-impairing working materials and xenobiotics as well as for the analysis of binding products (adducts) of hazardous substances with biological macromolecules (proteins and DNA).

As one of the most important techniques of liquid chromatography in analytical practice, high performance liquid chromatography (HPLC) has already been reviewed in a chapter in this collection of methods "Analyses of hazardous substances in biologi-

cal materials" (General Introduction to Volume 7). "The use of gas chromatography-mass spectrometry in biological monitoring" (General Introduction to Volume 10 of this series, which was recently published) also provided a comprehensive description of the physico-chemical fundamental principles of mass spectrometry and reviewed the instruments that are currently in use.

This chapter on "The use of liquid chromatography/mass spectrometry (LC/MS) in biological monitoring" is intended to provide considerable supplementary information on the above-mentioned current advances to that contained in the chapter on "LC-MS-coupling" (General Introduction to Volume 7, Section 5.5). The practical and instrumental principles of LC/MS will be described as well as some aspects of method development and optimisation with examples of applications for special substance groups. Wherever appropriate, we will refer the reader to the previous publications (HPLC, Vol. 7) and (GC/MS, Vol. 10) to avoid repetition.

The "biomonitoring methods" series already contains one LC/MS method ("Perfluorooctanesulphonic acid and perfluorobutanesulphonic acid in plasma and urine", Volume 10); this present volume includes two further methods ("Di(2-ethylhexyl) phthalate (DEHP) metabolites in urine" and "Monohydroxybutenylmercapturic acid (MHBMA) and dihydroxybutylmercapturic acid (DHBMA) in urine").

## 2 Principle of liquid chromatography/mass spectrometry (LC/MS)

As in the case of GC/MS, two independently functioning analytical systems are coupled in LC/MS. As a rule, the analyte(s) of interest is/are separated from the sample matrix by means of the liquid chromatographic system (e.g. HPLC or capillary electrophoresis (CE)) and introduced into the mass spectrometer as ions. Compared with e.g. UV detection, mass spectrometric analysis offers such a wealth of specific information on a substance that it is generally possible to determine structurally different analytes even if they have identical retention times, and to quantify them separately if desired.

Whereas the analyte is already in the vaporous state after separation by gas chromatography in GC/MS and only has to be ionised, in LC/MS an additional phase change is required, i.e. from the analyte dissolved in a liquid to the analyte in the gaseous phase (see Figure 1).

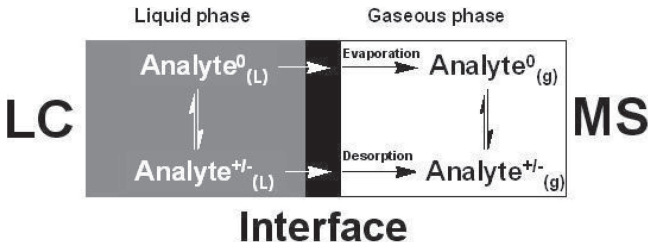


**Fig. 1.** Phase change and ionisation of an analyte in LC/MS according to [5]

These changes are achieved by specially designed interfaces. In general, LC/MS interfaces must be capable of facilitating three processes:

- evaporation of liquids and analyte molecules to vapours,
- ionisation of the uncharged analyte molecules in the gaseous phase or desorption of analyte ions from the liquid phase,
- evacuation of a strong stream of gas to maintain the high vacuum in the mass spectrometer.

Most LC/MS interfaces ensure that processes 1 and 2 proceed by the formation of an aerosol, as aerosol formation provides a highly effective enlargement of the surface area. The larger the surface area, the more readily the liquids and uncharged analyte molecules evaporate and the more readily analyte ions are desorbed from the liquid phase into the gaseous phase. Therefore improvement of the aerosol formation process poses a permanent challenge to manufacturers of LC/MS interfaces.

The third process, the evacuation of a strong stream of gas, poses a problem that can only be solved by great technical ingenuity, even in the case of GC/MS. Carrier gas flows of 0.5 to 2 mL/min are found there, which result in gas pressures of 1 to 2 bar at the end of the column. However, the use of a typical mass spectrometer requires a stable high vacuum of $10^{-5}$ to $10^{-6}$ mbar (see General Introduction to Volume 10 in this series). Overcoming the pressure difference when a liquid chromatographic system is coupled with a mass spectrometer presents an even greater challenge. As shown in Table 1, gas flows of more than 1000 mL/min may occur, depending on the type of LC column, the eluent(s) used and the flow rate. Only at very low flow rates (< 10 μL/min) are the gas flow rates for the LC/MS coupling in the same order of magnitude as those for the carrier gas in a GC/MS coupling.

Technically the problem of the pressure difference at the LC/MS interfaces of practical importance is solved by installing several pumping stages in series to attain a high vacuum in the mass spectrometer. The pump technology used for creation of the vacuum has already been presented in the General Introduction to Volume 10 of this series.

**Table 1.** Comparison of (carrier) gas flow rates depending on the type of the GC or LC column, the carrier gas or eluent and its flow rate [6]

| Column | Carrier gas/ Eluent | Flow rate [mL/min] | (Carrier) gas flow [mL/min] |
|---|---|---|---|
| GC (capillary) | Helium | 1 | 1 |
| GC (packed)/GC (CI) | Helium | 20 | 20 |
| LC (analytical) | Hexane | 1 | 184 |
| | Methanol | 1 | 593 |
| | Water | 1 | 1240 |
| LC (capillary) | Hexane | 0.01 | 1.8 |
| | Water | 0.01 | 12 |

If all LC/MS systems are taken into account, they have the following set-up in common:

- a sample injection or chromatographic system
- an LC/MS interface
- one or more mass filters
- a detector

The individual components will be described in detail and their importance for LC/MS will be discussed in Section 3. Wherever appropriate, we have chosen to provide a brief description and will refer readers to the principles presented in the previously published chapters (HPLC, Vol. 7) and (GC/MS, Vol. 10) to avoid repetition.

## 3 Set-up of liquid chromatography/mass spectrometry coupling

### 3.1 Sample injection or chromatographic system

Three main techniques can be used for sample injection into an LC/MS system: a syringe pump, high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Independent of the technique used, sample injection must be performed as stipulated in the General Introduction to Vol. 7. It is particularly important to note that most LC/MS interfaces do not function well when samples contain high electrolyte concentrations (non-volatile salts, certain acids and alkalis) and matrix components. Under the most favourable conditions the performance of the instrument is impaired, and in the worst-case scenario severe damage to the relevant LC/MS interface may be caused. In this case it is advisable to precipitate proteins by the addition of organic solvents, as the sample is also simultaneously diluted. Alternatively, ultrafiltration may be carried out with subsequent dilution of the filtrate if necessary. Further currently employed procedures for sample processing are discussed in detail in Section 3.1.2.2 of this chapter.

#### 3.1.1 Syringe pump

The syringe pump introduces the sample directly into the MS and, as a rule, it is supplied when the system is purchased. Flow rates from several nL/min up to several mL/min can be selected, depending on the size of syringe used, but 5 to 50 μL/min are normal. The syringe pump continuously infuses an analyte mixture onto the system during sample injection, but the individual components of a complex sample matrix are not separated off. In this case the specificity of the analysis is not based on the combination of a specific retention time with the results of the mass spectrometric analysis, but solely on the substance-specific information provided by the mass spectrometer. Therefore the syringe pump is suitable for two applications: the mass spectrometric analysis of reference substances (standards) and investigation of previously cleaned-up fractions.

In the first case the chemical structure is already known, generally by characterisation using another physico-chemical procedure (e.g. nuclear resonance spectroscopy). These substances serve as standards e.g. for the development of LC/MS methods that involve a chromatographic separation of the analyte(s) from the sample matrix. The pure analyte itself (insofar as its chemical structure is known) or its analogue labelled with heavy isotopes is often selected to serve as a standard ("internal standard"). Infusion of the solution containing the relevant pure substance with the aid of the syringe pump yields initial information on the behaviour of the reference substance (or that of the analyte) at the LC/MS interface and in the mass spectrometric analysis. The analyte ion yield can be qualitatively and quantitatively investigated and optimised by variation of the infusion conditions (e.g. flow rate, solvent composition of the sample) and by adjustment of the instrumental parameters for the LC/MS interface and mass spectrometer. The results obtained form the basis for the set-up of an "on-line" LC/MS procedure (see also Section 4.2 "Development and optimisation of methods" under "Tuning").

The second application is to facilitate the investigation of previously cleaned-up fractions. Such fractions can be obtained e.g. by liquid-liquid extraction, by solid phase extraction or in the form of an eluate from a preparative chromatographic procedure. In this case the specificity of the analysis is based on the selective enrichment of the analyte by extraction or chromatography and on the results of the mass spectrometric analysis. The "off-line" LC/MS methodology presented here has proved successful in clarifying the structure of unknown compounds as well as in measurement procedures with a high throughput in pharmaceutical analysis [7].

When a syringe pump is used, a technical distinction is made between low flow direct infusion and high flow infusion.

In the case of low flow direct infusion the syringe pump is directly connected to the LC/MS interface. This configuration is preferred for small sample volumes; selection of low flow rates from the nL/min range up to the lower µL/min range permits complete mass spectrometric analysis with high sensitivity, while sample consumption is kept to a minimum.

An LC system is required for infusion at higher flow rates. In this configuration the LC system is connected to the LC/MS interface by means of a T-junction. The syringe pump is connected to the second inlet on the T-junction. It is important to check that all the connections are tightly sealed, as even tiny leaks can have dramatic effects on the quality of the results.

The LC system is normally operated at a flow rate of 100 µL/min to 1 mL/min. The syringe pump infuses the sample solution into this constant stream of solvent (typical flow rates 1 to 10 µL/min). The resulting dilution of the sample solution depends on the ratio of the selected flow rates to each other. Good results can still be achieved in this configuration with concentrations of 1 pg/µL (after dilution).

Adverse effects on the LC/MS interface due to high electrolyte concentrations in the sample solution can be greatly reduced or even eliminated by dilution. However, some LC/MS interfaces achieve high ion yields only when flow rates are high, and an infusion at a high flow rate is then a prerequisite for correct analysis. Finally, the parameters of an on-line LC/MS method, such as different solvent compositions and

different flow rates, can be simulated very well and varied rapidly in this configuration. The effects of each variation on the analyte ion yield in the LC/MS interface and the mass spectrometer can be investigated qualitatively and quantitatively. The information obtained from such experiments forms an important basis for setting up an on-line LC/MS method.

### 3.1.2 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is the most widely employed separation technique for routinely applied on-line LC/MS methods [5, 8]. The principles of HPLC have already been described in detail in this "Analyses of hazardous substances in biological materials" collection of methods (General Introduction to Volume 7). Therefore only special aspects of HPLC when combined with MS will be discussed here. We have chosen a general approach and in the following sections we will deal with some specific points, e.g. individual LC/MS interfaces in combination with HPLC.

#### 3.1.2.1 MS-compatible HPLC systems

In theory all the HPLC phase systems mentioned in Volume 7, such as reverse phase chromatography, ion exchange chromatography and affinity chromatography, can be considered for use in an LC/MS configuration. In practice, however, their applicability is limited because compatibility with the relevant LC/MS interface is essential. Significant factors that influence the required compatibility are the flow rate at the end of the column and the composition of the eluents there, which, as stated above, may not contain high concentrations of electrolytes. In particular, reverse phase chromatography (RPC) meets these requirements and is therefore compatible with all known LC/MS interfaces. Therefore about 80% of all LC/MS methods with an HPLC application use RPC [5].

The inner diameter of the HPLC column has proved a useful parameter for classifying the HPLC columns used for LC/MS. Several groups can be distinguished: columns for conventional HPLC (preparative and analytical: wide-bore and normal-bore), narrow-bore columns and columns for capillary liquid chromatography (micro-bore and nano-bore). Table 2 shows typical inner diameters and flow rates.

Although preparative HPLC columns are included in this list for the sake of completeness, their importance for LC/MS is negligible. In contrast, analytical (normal-bore) and narrow-bore columns are prevalent in routinely used on-line LC/MS systems; there is also a growing tendency to use micro-bore and nano-bore columns for LC/MS applications [8]. Table 3 gives the most important characteristics of the various types of columns.

When the figures in Table 3 are considered, it is evident that miniaturisation of HPLC columns is accompanied by reduction in the amount of solvent consumed (smaller dead volume of the column, lower flow rates), less sample consumption

**Table 2.** Classification of HPLC columns for LC/MS according to their inner diameter [8]

| Type of column | Inner diameter [mm] | Flow rate [mL/min] |
|---|---|---|
| Preparative (wide-bore) | >4.6 | >3 |
| Analytical (normal-bore) | 3–4,6 | 0.5–3 |
| Narrow-bore | 1–2 | 0.02–0.3 |
| Micro-bore | 0.15–0.8 | 0.002–0.02 |
| Nano-bore | 0.02–0.1 | 0.0001–0.001 |

**Table 3.** Typical characteristics of HPLC columns according to Tomer et al. [9] and Abian et al. [8] (uniform column length: 25 cm)

| Inner diameter [mm] | Column dead volume [μL] | Flow rate [mL/min] | Injection volume [μL] | Relative concentration at detector |
|---|---|---|---|---|
| 4.6 | 4100 | 1 | 100 | 1 |
| 2 | 783 | 0.2 | 19 | 5.3 |
| 1 | 196 | 0.047 | 4.7 | 21.2 |
| 0.32 | 20 | 0.0049 | 0.485 | 206 |
| 0.05 | 0.49 | 0.00012 | 0.012 | 8459 |

(smaller injection volume) and a dramatic enhancement in the detection sensitivity (increase of the relative concentration at the detector by three powers of 10). These figures present clear arguments in favour of increasing the use of capillary liquid chromatography in LC/MS, which has not yet occurred in routine analysis to date.

However, technical and practical aspects must also be taken into consideration when HPLC columns are selected for LC/MS. For instance, analytical and narrow-bore columns can be operated with conventional HPLC systems, whereas existing systems have to be converted in order to perform capillary liquid chromatography [10, 11]. Change parts are commercially available to convert the common HPLC systems.

In addition, it should be noted that a method using an analytical or narrow-bore HPLC column that has already been established with another detector can generally be more rapidly modified to a LC/MS method. The already developed chromatography can often be taken over; the existing method must only be checked for compatibility with the LC/MS interface, and optimised if necessary.

Moreover, the quality of the HPLC columns used plays an important role. A large selection of analytical and narrow-bore columns packed with various materials is available from different commercial suppliers. They all provide a test chromatogram showing the most important chromatographic indices. This information indicates the batch conformity of the packing material and the compliance of its processing with

requirements. The guarantee of conformity is of critical importance for an established LC/MS routine procedure that is set up for high sample throughput, as otherwise fluctuations in the quality of the packing material would entail time-consuming optimisation of the existing chromatography each time the column had to be exchanged.

In contrast to the analytical and narrow-bore HPLC columns, only a small selection of micro-bore and nano-bore columns are available from a few commercial suppliers [8]. One reason for the limited range is that the columns already available are undergoing continual development. Under these circumstances and due to the relatively high acquisition costs, many users have opted to prepare their own packed micro-bore and nano-bore columns according to various procedures [9, 12–17]. On the one hand, this has the advantage that the chromatography can be very rapidly and flexibly modified and optimised during development of an LC/MS method as part of research work. On the other, as discussed above, possible fluctuations in the quality of in-house packed HPLC columns may have negative effects on an LC/MS routine procedure once it has been established.

The technically possible injection volume also represents an important criterion for the choice of HPLC columns for LC/MS. Whereas analytical and narrow-bore HPLC columns may permit input of large sample volumes containing low analyte concentrations at µL levels using standard sample injection systems (General Introduction, Volume 7, Section 5.1), the injection volumes for capillary liquid chromatography are in the nL range. This requires the use of specially adapted sample input systems and prior enrichment of samples. Despite such difficulties various techniques [8] can be utilised to enrich the samples at the beginning of the column in order to enable µL-level sample volumes with low analyte concentrations to be injected onto micro-bore and nano-bore columns. However, overloading may easily result on account of the low maximum capacity of micro-bore and nano-bore columns, especially when the analyte(s) are contained in complex matrices. The use of a narrow-bore pre-column for on-line concentration of the sample and subsequent desorption of the enriched fraction for capillary liquid chromatographic analysis offers a possible solution [18, 19].

When the technical and practical aspects of the selection of HPLC columns for LC/MS are taken into consideration, it is understandable why many users [8] choose the analytical and narrow-bore HPLC column options: existing HPLC systems can be harnessed without changing the configuration, standard HPLC methods already established with other detectors can be readily adapted to LC/MS procedures, high quality, standardised column material is commercially available, and it is possible to inject large sample volumes with low concentrations of the analyte in complex matrices without problems using standard sample injection systems. If we compare the frequency of use of analytical and narrow-bore HPLC columns, then the narrow-bore HPLC columns are highly popular [8]. One reason is the relative sensitivity at the detector, which is enhanced by about a factor of 10 in comparison with that attained by analytical columns (Table 3), another reason may be the typical flow rates of 50 to 500 µL/min, which are very compatible with some of the commonly employed LC/MS interfaces.

Although the use of analytical and narrow-bore HPLC columns reflects the current state of the art, the future belongs to capillary liquid chromatography and miniaturised LC/MS systems. A decisive reason for this development is the excellent sensitivity of this HPLC technique. Thus, for example, "on-column" detection limits have been recorded at the low femtomole level for the determination of peptides from cell culture supernatant liquid [18]. New developments apply a high direct current (20 to 50 kV) over the entire length of the micro-bore and nano-bore column to diminish a possible band broadening and for an additional enhancement of the separation power from a maximum of 40 000 to 65 000 theoretical plates in the original capillary liquid chromatography [16] to 200 000 theoretical plates [8, 20]. The increase in separation power is attributed to an improvement in the mass interaction between the stationary and the liquid phases. The new analytical technique is used in two types of application: capillary electrochromatography (CEC) and pseudo-capillary electrochromatography (pCEC). Like capillary electrophoresis, the liquid phase is transported solely by electro-osmosis in CEC [20]. As in classical HPLC, additional flow is generated by pressure in the case of pCEC [21, 22].
A greater availability of miniaturised HPLC systems, a broad range of commercially available high-quality micro-bore and nano-bore columns at reasonable prices and the development of robust methods, especially LC/MS procedures to detect low analyte concentrations in complex matrices – in particular with regard to analysis in biological materials – will be of decisive importance for the future success of the different variations of capillary liquid chromatography in routine laboratory practice.

### 3.1.2.2 Sample preparation for routine analysis (multi-dimensional HPLC)

The use of LC/MS methods often permits sample preparation to be reduced to a minimum, as urine and blood samples can be analysed as aqueous solutions without further derivatisation after separation of the protein components by precipitation using the HPLC solvents methanol or acetonitrile and subsequent centrifugation [23–25]. Thus losses of the analyte due to sample processing are reduced and sensitivity is simultaneously improved. However, matrix effects may sometimes have adverse effects that influence the chromatographic separation (e.g. broadening of the signals). In addition, a matrix-rich analytical solution may reduce (but rarely enhance) the ion yield. In most cases it leads to more rapid build-up of contamination in the complete system. As a rule, the consequences are lower sensitivity as well as diminished robustness of the method. This leads to shorter maintenance intervals in order to restore the capability to perform sensitive measurements. Thus the time saved by reducing sample preparation and the advantage of higher sensitivity due to lower losses of the analyte may be nullified [26–29]. In a review Mallet et al. describe a method of quantifying ion suppression or ion yield enhancement and point out some ways of avoiding these effects [30]. In addition, the authors explain pH effects and their consequences on the ion yield of various analytes and on ionisation polarity.
If sample preparation is extremely minimised, samples should be filtered and the HPLC columns should be protected from matrix contamination by guard columns.

Blood and urine contain many salts and organic compounds that are readily soluble in water; these compounds are generally eluted when the proportion of organic solvent is low, and they therefore suppress the ionisation yield of an analyte that is eluted under the same conditions. By selecting the HPLC conditions (type of column, gradient elution) so that the analyte leaves the column with as high a proportion of organic eluent as possible, improvement in sensitivity is obtained; in addition it is possible to use a switch valve to divert the eluate that does not contain the analyte to a waste container. This diminishes contamination of the mass spectrometer.

Further procedures have been developed to increase the sensitivity and degree of automation of LC/MS analyses. Zell et al. published a method for analysing larger volumes without overloading the analytical HPLC column and contaminating the entire analysis system and thus nullifying the improved sensitivity [19, 31, 32]. This system, now known as column-switching, carries out a shortened form of column chromatography to achieve enrichment and pre-cleaning of the sample with the aid of a 6-port valve and a "trap" column. For this purpose up to several hundred microlitres of test solution are loaded onto the trap column together with an eluent containing as high a proportion of water as possible. More lipophilic analytes are bound on the column, whereas polar components are washed into a waste vessel. After the washing procedure the analytes are eluted from the trap column onto the analytical column with the aid of a gradient pump and an eluent with a higher organic proportion. Then the actual chromatographic separation can be performed as described above. Applications of this principle for analyses in biological materials can be found in Koch et al., Brink et al. and Kellert et al. [33–35]. As several millilitres of sample material are normally available for analysis, column-switching represents a convenient method of attaining very low detection limits.

The transfer of the target analytes from the trap column to the analytical column can be carried out in the same direction as the flow onto the trap column or the direction of flow can be reversed in the so-called "back-flush" procedure (see Figure 2). As a rule, the back-flush technique leads to transfer of the target analytes onto the analyti-

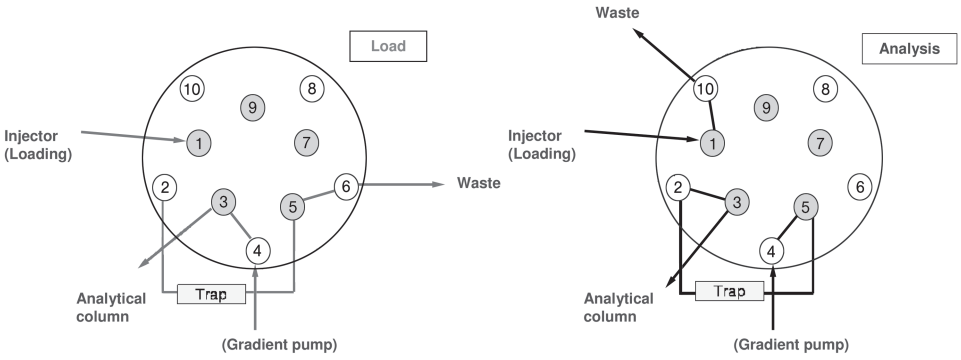


**Fig. 2.** Schematic illustration of a column-switching system

cal column as sharply defined bands, with consequential advantages for chromatographic separation and quantitative determination.
Other variations of column-switching using only one column (in this case the trap column is used for sample preparation and serves as the analytical column too) as well as the use of 3 columns (with two trap columns in parallel or two analytical columns in parallel) are described in reviews of this subject [36–38].

### 3.1.2.3 Trap columns

Trap columns that permit direct injection of material containing protein without previous protein precipitation have now been specially developed for analyses in biological materials (urine, blood, plasma, sputum). Depending on the analytical investigation, only hydrolysis (to cleave phase II conjugates) and/or pH adjustment have to be carried out before injection.
Separation of the protein matrix by these biocompatible trap columns, which have become commercially available only in recent years, is achieved by means of various physical separation principles. Reviews of this field [36, 38, 39] provide comprehensive overviews. The trap columns mentioned below all have the advantage that, depending on the demands of the analysis, up to several mL of urine or up to 100 µL of serum/plasma can be introduced in one injection without chromatographic problems. Depending on their type and the application, the useful life of these trap columns is given as several hundred cycles, and this is also attained. Centrifugation of the samples before injection prolongs the useful life of trap columns. The duration of a chromatographic separation run depends on the type of trap column used, but a fully automated run is prolonged only by between 0.5 and at most 10 minutes and considerable time is saved in sample preparation.

*Restricted access media (RAM)*
The term "restricted access media" (RAM) describes a family of column materials that permits direct injection of biological fluids, in which only low-molecular substances (<15 to 20 kDa) can interact with the active groups of the column material (RP materials with C4, C8, C18, nitrile or phenyl groups). Conversely, high-molecular compounds are denied access to active sites on account of their size (pore size or chemical barriers). In addition, a hydrophilic coating on the outer surface of the column material prevents adsorption of the protein matrix [39, 40].
RAM phases that exclude the protein matrix due to the specific pore size of the basic material are commercially available in different variations. ISRP ("Internal Surface Reversed Phase") columns (from Regis Technologies) are inaccessible to molecules with a size greater than 20 kDa and retain hydrophobic molecules on a tripeptide phase (glycine-L-phenylalanine-L-phenylalanine) in the internal pores (e.g. GFF and GFF II) mainly by means of interaction with the $\pi$ electrons [41, 42]. These columns are supplied in the dimensions of both pure trap columns (1 cm×3 mm) and analytical columns (up to 25 cm×4.6 mm). ISRP columns have proved especially efficient in the direct analysis of serum [37, 40] but applications in the analysis of

urine have also been reported [42]. ADS (alkyl-diol-silica) phases (Merck) with a pore size of 6 nm exclude molecules with a size greater than approx. 15 kDa. Internal coatings with C4, C8 and C18 reverse phases are currently obtainable [39]. These ADS columns have been specially developed as trap columns (2.5 cm×4 mm) and are at present the most popular RAM phases for biological fluids. The wide range of applications, both in serum and in urine as well as in other biological fluids (sputum, microdialysate, milk, etc.), is described comprehensively in the literature [36, 38, 40]. The analyses of the phthalate metabolites in urine [35] and serum [43] give currently valid examples (see also "Di(2-ethylhexyl)phthalate (DEHP) metabolites" in this volume). The use of the ADS phase has been limited in particular by the fact that only C4, C8 and C18 classical reverse phases have been commercially available as internal coatings to date, and retention of even moderately polar target analytes is poor. The ChromSpher 5 Biomatrix column (Chrompack/Varian) with a pore size of 13 nm retains small molecules by hydrophobic interactions with phenyl groups. However, the reported range of application of this column is rather narrow [38].

A second type of RAM phase achieves the exclusion of proteins and other macromolecules mainly or additionally by means of a chemical barrier. In SPS ("semipermeable surface") phases (Regis Technologies) proteins are excluded by a semipermeable polyoxyethylene coating of the silica material, while retention occurs in the underlying nitrile, phenyl, C4 or C18 phases that are accessible only to smaller molecules. Like ADS columns, SPS columns are remarkable for their robustness, whereby SPS columns offer a wider range of reverse phase retention [44]. As in the case of GFF columns, SPS columns are available in the dimensions of both pure trap columns (1 cm×3 mm) and analytical columns (up to 25 cm×4.6 mm). The basic silica material (C8 and C18) or the hydrophobic basic polymer material of BioTrap and BioTrap MS columns (Chromtech) is coated with the human plasma protein $\alpha_1$-acid glycoprotein (AGP). The BioTrap MS column can be used in a relatively wide pH range from 2 to 10. BioTrap columns are reported to be especially suitable for analysis in serum and plasma on account of the coating with AGP [38, 45]. Capcell PAK columns (Shiseido/Phenomenex) consist of a silica material with a silicone monolayer coating, whereby active silanol groups are protected by alkyl chains. Polyoxyethylene chains prevent the adsorption of macromolecules. Depending of the type of Capcell, retention of molecules of less than 20 kDa is achieved by interaction with C1, C8, C18, phenyl, CN, $NH_2$ or cation-exchanger (SCX) groups. Eight different types of Capcell columns are now commercially available, some of which are also suitable for retention of relatively polar compounds (e.g. MG-II, AQ types). In addition, the Capcell columns are remarkable for their applicability over a wide pH range (1 to 10). Column dimensions range from purely trap columns (1 cm×4 mm) to analytical columns (up to 25 cm×4.6 mm). Several publications confirm the wide-ranging applicability of Capcell columns for the analysis of urine, serum and plasma samples in one-dimensional and multi-dimensional separation systems [38, 46–49]. Another type of column, which is specially marketed for direct injection of serum samples, is Supelcosil Hisep (Sigma-Aldrich). This silica-based material is protected from protein bonding by polyethylene oxide, while low-molecular compounds are

bound by phenyl groups [38]. Column dimensions from 2 cm×4.0 mm to 25 cm × 4.6 mm are available.

*Large particle support (LPS)*

Another approach that enables direct injection of biological fluids is the use of column materials with relatively large particle diameters (25 to 60 μm). "Turbulent flow chromatography" (TFC) makes use of columns with small inner diameters (between 0.5 and 2.1 mm) that are operated at high flow rates (3 to 5 mL/min). This changes the laminar flow profile into a turbulent flow profile. Analytes with a molecular size of <1000 Da can be retained in the hollow spaces in this way. Conversely, macromolecules and salt clusters are not retained. TFC leads to a very good exchange between the stationary and the mobile phase with the result that the concentrated analyte can be effectively transferred from this trap column to the analytical column. This technique makes further sample pre-treatment unnecessary, especially in the case of matrices containing protein (plasma, tissue homogenates). Detailed descriptions can be found in [50–59]. On principle, two types of column material are used in this application: (1) conventional silica material with e.g. C2, C8, C18 or phenyl coating and (2) polymer material (to date divinylbenzene-N-vinylpyrrolidone copolymer). In general, all the column materials known in "off-line" SPE can also be used in TFC. Both types of column permit several hundred plasma injections of 100 μL without recognisable deterioration in the analytical performance. This applies only if the sample is introduced under turbulent flow conditions. Under laminar flow conditions protein accumulation soon renders the column unusable [60]. It is also remarkable that, on account of the high flow rates, introduction and washing cycles of less than 1 min are sufficient before the sample is transferred to the analytical column and analytical separation is achieved by means of column-switching and by a flow rate reduction to a range of 0.25 to 0.5 mL/min.

Oasis columns (Waters) have been developed specially for the requirements of TFC and are now offered in various modifications and in column dimensions from 2 cm×2.1 mm up to 2 cm×4.6 mm. Oasis HLB (hydrophilic lipophilic balance) is especially suitable for the retention of lipophilic molecules by divinylbenzene groups, whereas moderately polar to hydrophilic molecules are retained by N-vinylpyrrolidone groups. Thus Oasis HLB covers a much broader analytical spectrum than conventional C18 materials. Other Oasis modifications, such as Oasis MCX, Oasis WAX, Oasis MAX and Oasis WCX, combine the RP phase properties of HLB with anion and cation exchanger characteristics of varying strength. Therefore at present Oasis columns provide the widest range of retention mechanisms for on-line sample preparation with trap columns. Numerous publications describe the use of Oasis columns for on-line extraction of analytes from biological fluids [38, 61–66].

A second LPS technique that is also quite new is the use of monolithic columns for direct injection of biological fluids. In this case too (like column material with large particle diameters) sample injection can be carried out at high flow rates without generating high pressure on account of the high permeability of these columns. The flow rates are 5 to 10 times higher than in the conventional application of monolithic columns. Under these conditions the higher-molecular protein matrix is also eluted

with the solvent front. Applications for plasma as well as urine samples have been described [38, 67, 68]. At present, the Chromolith Flash RP-18e column (Merck) seems especially suitable for use as a trap column (dimensions of 2.5 cm×4,6 mm) [69].

*Further on-line techniques*
Some further on-line techniques that have also been made commercially available recently must be mentioned in this context. On-line solid phase extraction (SPE) that can be integrated into the software and hardware of the common LC/MS systems appears promising, as it permits fully automated analysis of plasma and urine samples without further processing. In this case the SPE columns are automatically replaced by the instrument after being used once or several times, depending on wear and tear. This system generally serves to separate undesirable matrix components rather than to enrich the analyte. The first publications indicate that this advance is promising and readers should keep an eye on future developments [70].
When very hydrophilic compounds (glucuronide and sulphate metabolites) are analysed with a higher proportion of organic eluent, it may prove impossible to elute the analyte by HPLC separation with conventional C18 materials. An isocratic chromatographic separation must be performed with a very low percentage of organic solvent (<5%), e.g. to prevent elution of the analyte with the dead volume of the column. Acetonitrile or other organic solvents that form a maximum azeotropic mixture with water can be added to the eluate through a T-junction after chromatographic separation of the analyte. This facilitates the transition of the analyte molecules into the gaseous phase, and despite dilution due to addition of the solvent, the ion yield is enhanced as shown by the example of ethylglucuronide [71]. It is also possible to increase the ion yield by reducing the HPLC flow rate at the end of the separation column. For this purpose the eluate can be divided into portions after the HPLC column and a smaller portion can be transferred to the LC/MS interface. Although much less analyte reaches the mass spectrometer, this loss is more than compensated by the improved ion yield. We refer readers to the study carried out by Gangle et al. for further details [72].

### 3.1.3 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a micro-separation technique for organic compounds that are readily ionised in solution. It is based on application of a high direct current (15 to 30 kV) to the entire length of a "fused-silica" capillary column (inner diameter: ≤100 µm) filled with buffer solution. This leads to electro-osmotic flow of the solution at a rate of 0 to 200 nL/min with a drop-like profile. The different migration rates of the ions in the solution under the influence of the electric field can result in a very effective separation of the analyte(s) from the matrix. Technically five separation principles (modes) are employed: capillary zone electrophoresis (CZE), isotachophoresis (ITP), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE) and capillary isoelectrofocusing (CIEF) [73, 74].

The theoretical advantages of this separation technique are minimisation of sample volumes, low consumption of solvents, short separation times, high resolution and simple method development. At present, however, CE is still in the transition phase from research to routine practice. Potential clinical users are hoping for the development of instruments that are completely suitable for routine use and that permit a high sample throughput as well as for ready-to-use applications in the form of user-friendly kits containing all the necessary components [73].

With regard to LC/MS, efforts have been made to use CE as a separation system since the 1980s [75–77].

The main problem in coupling CE with a mass spectrometer is posed by the low flow rates. They are typically in the range of $<1$ to 100 nL/min. In contrast, the most frequently used interfaces are operated at flow rates ranging from 1 to 200 μL/min. Various measures can be taken to compensate for this difference: on the one hand the flow rate at the end of the capillary can be increased by feeding external liquid ("sheath-flow" CE interfaces and "liquid junction" CE interfaces), on the other hand hopes are pinned on a general miniaturisation of interfaces ("sheathless-flow" CE interfaces) [20]. As described in Section 3.1.1 the infeed of liquid leads to dilution of the analyte and to a possible loss in sensitivity. But miniaturisation of the interfaces requires considerable technical ingenuity and users need special training to handle these LC/MS systems, so neither of the two approaches has yet born fruit in efforts to make this technique suitable for routine analysis.

The low maximum loading capacity of fused silica capillary columns places another obstacle in the path towards routine use of CE, especially with regard to analyses in biological materials. As described for capillary liquid chromatography (Section 3.1.2) columns can easily become overloaded, particularly when the analyte(s) is/are present in complex matrices. In this case too, an attempt can be made to use "on-column" enrichment techniques so that samples with volumes at the μL level with low analyte concentrations can be introduced [76, 78].

In their review article of 1995 Cai and Henion [75] discussed the low concentration-dependent sensitivity of the CE/MS coupling ($\geq 10^{-5}$ M) that hindered the use of CE/MS for routine analysis at that time. However, in their opinion the technique would be improved in the thereafter, resulting in wider distribution among users. In 2002 Gelpí [20] came to the conclusion that these expectations had not yet been fulfilled. Thus CE/MS coupling has still not become a routine analytical technique – also with regard to analysis in biological materials – but remains an interesting field of research.

## 3.2 LC/MS interfaces

As already discussed above, the interface plays a central role in LC/MS coupling. In the preceding (almost) forty years different technical solutions have been offered to overcome the problem of transforming analyte molecules or ions present in the liquid phase to analyte ions in the gaseous phase. Depending on whether the analyte molecule is first enriched and then ionised (sampling/ionisation) or first ionised and

then enriched (ionisation/sampling), the interfaces employed for on-line LC/MS procedures can be assigned to one of two categories [20]. The first category comprises the "moving wire/moving belt" interfaces, the "particle beam" interface, the "direct liquid introduction" interface and the "continuous flow fast atom bombardment" interface. The second category consists of atmospheric pressure ionisation (API) sources, such as electrospray ionisation (ESI), the "atmospheric pressure chemical ionisation" (APCI) source and the "atmospheric pressure photo-ionisation" (APPI) interface as well as its precursor the thermospray interface.

### 3.2.1 Classical LC/MS interfaces

The interfaces mentioned in the first category and the thermospray interface belong to the classical LC/MS interfaces. These classical LC/MS interfaces have greatly declined in importance for routine analysis in recent years since the introduction of robust commercially available atmospheric pressure ionisation sources. They are still suitable for discrete applications in some cases, but manufacturers of some interface types have discontinued their production, e.g. thermospray interfaces [5]. For these reasons classical LC/MS interfaces will not be discussed in detail in this section. Interested readers are referred to publications on the basic studies and reviews given in Table 4.

### 3.2.2 Atmospheric pressure ion sources

It is characteristic for atmospheric pressure ion sources that analyte molecules are ionised at atmospheric pressure and then subsequently enriched and transferred to a mass spectrometer. Three methods of ionisation are possible for routine analysis and they form the physico-chemical basis for the design of commercially available instrumental systems: the application of high voltage electric fields (ESI), electrical discharge (APCI) and irradiation with a UV source (APPI). Whereas the number of publications describing classical LC/MS interfaces for analysis has decreased in re-

**Table 4.** Literature overview of classical LC/MS interfaces

| LC/MS interface | Basic studies | Reviews |
|---|---|---|
| Moving wire/moving belt | [79, 80] | [81, 82] |
| Particle beam | [83, 84] | [85, 86] |
| Direct liquid introduction | [2–4, 87–89] | [90, 91] |
| Continuous flow fast atom bombardment | [92, 93] | [94, 95] |
| Thermospray | [96–99] | [100–102] |

cent decades, the number of articles on the use of ESI and APCI sources has risen sharply, with studies on the APPI interface reinforcing this trend since 2000 [6, 103]. The three types of LC/MS interface will be presented in detail below, the fundamental physico-chemical processes will be introduced, and the schematic instrumental set-up (with variations if applicable) will also be explained.

### 3.2.2.1 Electrospray ionisation (ESI)

The first applications of ESI were reported by Fenn et al. [104–106] and by Aleksandrov et al. [107] independently of each other in the mid-1980s. Figure 3 shows the electrical circuit on which ESI is based.

A sharply pointed steel capillary (needle) serves as one electrode, and a collector (aperture plate) functions as the counter-electrode. A high potential of several kilovolts is applied between the two electrodes, whereby oxidation occurs at the needle and reduction at the collector. The eluent from the LC completes the electrical circuit. A prerequisite for detection by means of ESI-MS is that the analyte must be present as an ion in the eluent. The applied electric field ensures a partial separation of the positively and negatively charged ions in the eluent by partly penetrating the liquid surface at the tip of the steel capillary. In the positive ion mode shown here, positively charged ions are enriched at the tip of the needle, while negatively charged ions are forced back into the interior of the steel needle. In the negative ion mode the poles are reversed and the contrary effect is achieved.

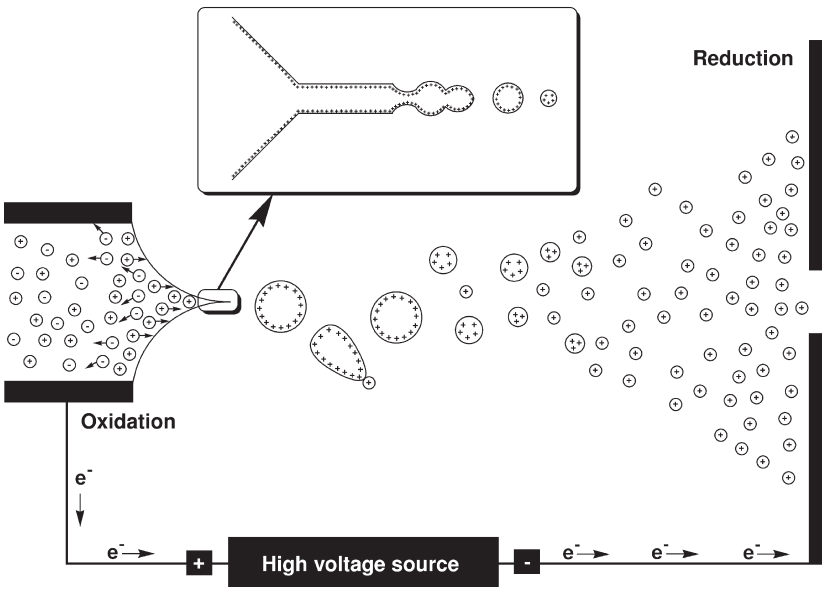


**Fig. 3.** The electrospray process (according to [108])

The surface tension of the liquid is overcome due to the mutual repulsion at the surface and the simultaneous attraction of the positively charged ions to the counter-electrode under the influence of the electric field; a cone of liquid forms (the Taylor cone) with its tip being the last stable point that joins a thin thread of liquid, which in turn breaks down to individual charged droplets (see Figure 3, enlargement). The following equation gives the electric field strength $E_0$ required to initiate the electrospray process:

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

whereby $\gamma$ is the surface tension of the liquid, cos 49° is half the angle of the Taylor cone, $\varepsilon_0$ stands for the electric field constant and $r_c$ is the radius of the steel capillary. The surface tension of the liquid has a remarkable influence on the process. Liquids such as water have such a high surface tension that the vacuum field strength required for the electrospray process can lead to electrical corona discharges, especially in the negative ion mode. Special precautions must be taken to suppress these undesirable discharges when ESI is used [109]. A stable electrospray that forms a Taylor cone requires LC flow rates of 0.5 to 5 µL/min [6] and ion concentrations of $\geq 10^{-5}$ up to $< 10^{-3}$ M at the tip of the needle, with ion concentrations of approx. $10^{-5}$ M representing optimum conditions for charge separation [108].

A counter stream of hot nitrogen (typical temperatures: 200 to 300 °C, typical gas flow: 1 to 12 L/min) evaporates liquid from the tiny charged droplets formed by the spray process on their path from the needle to the collector. This diminishes the droplet size and the surface charge density increases. If the surface charge density exceeds the critical value of $10^8$ V/cm$^3$ (the "Rayleigh limit") the drops are subjected to tear-shaped deformation due to the repulsive forces of like charges, and a stream of fragments is emitted [110, 111]. The resulting total surface of the fragments is higher than that of the original droplet. If it is assumed that the initially present charges have a statistically uniform distribution on the resulting fragments, then the surface charge density is reduced and falls below the critical Rayleigh limit again.

Depending of the size of the original drop, the released fragments may be smaller droplets, which are further evaporated and in turn emit fragments when the Rayleigh limit is exceeded anew. This process proceeds until no more liquid can be evaporated and the ions either crystallise as solids or pass into the gaseous phase. This description of the electrospray process is known in the literature as the "charge residue model" (CRM) [1, 112, 113]. It is also possible that ions are directly emitted from the liquid phase into the vapour phase from droplets with a radius of 10 to 20 nm. The surface charge density required for this is below the Rayleigh limit. This is known as the "ion evaporation model" (IEM) [114, 115].

Which model or which combinations of both models best explains the electrospray process is the subject of current scientific discussion. However, IEM seems to better reflect the behaviour of smaller molecular ions during ESI, whereas CRM provides a very good description of the physico-chemical processes in the case of larger molecular ions, e.g. globular proteins [108].

ESI is notable for three characteristics. Firstly, it is a very mild ionisation technique that is highly efficient in generating protonated (or deprotonated molecular ions in the case of the negative ion mode) from polar, even thermally labile, compounds with a high molecular weight. Secondly, it generates multiply charged ions of the $[M + nH]^{n+}$ type (analogue ions are observed in the negative ion mode) from molecules with multiple basic groups. Thirdly, the molecular ions formed directly reflect the acid-base equilibrium in the LC eluent because they are formed by protonation/deprotonation of the molecules in solution or by formation of adducts with solvent ions. Therefore ESI is especially suitable for the detection of compounds that can be readily ionised in solution [6].

The formation of multiply charged ions has particularly wide-reaching consequences for the use of ESI. Mass spectrometers measure mass-to-charge ratios (m/z). Signals of molecular ions bearing multiple charges appear in a lower mass range than the singly charged molecular ion. Thus, for example, a protein with a 5-fold charge and a molecular weight of 8 kD yields measurable signals at 1600 m/z (m: 8000/z: 5). Whereas the signal of a singly charged molecular ion would not be detectable at a measurement range of 50 to 2000 m/z by the mass spectrometer, the signals of the protein bearing a 5-fold charge can be readily detected after ESI. As a rule, however, a molecular ion with a 5-fold charge does not generate one single signal, but a series of signals from differently charged ions emanating from the same molecule ($[M + nH]^{n+}$; n: 1, 2, 3, 4, 5, 6, …). The real molecular weight of the analyte can be calculated from the distribution and intensity of the observed signals with the aid of special computer programs known as "deconvolution" software. Thus a quadrupole mass spectrometer is capable of determining the molecular weights of compounds up to 50 kDa with a mass deviation of less than 0.01% [116].

Figure 4 shows the schematic set-up of an electrospray interface ("curtain gas" type). The electrospray process comprehensively described above takes place between the needle and the aperture plates. An additional heated glass capillary installed between the aperture plate and the ion optics often aids focusing and final desolvation of the generated ions. The ion optics (skimmer, octopole, lenses) focus the ion beam before it enters the mass spectrometer. The high vacuum necessary for the mass spectrometer is achieved by several vacuum chambers arranged in series. The pump technology used to create the vacuum has already been described in the General Introduction to Volume 10 of this series.

The linear arrangement of the needle and the aperture plates shown here permits optimal entry of the ions generated by the electrospray process and thus enables the greatest possible sensitivity, but at the same time it poses the risk of clogging due to deposits of liquid(s) or solid(s) from the spray. Various commercial suppliers have attempted to overcome this problem by alternative arrangements of the needle and aperture plates or by special inlet systems [6, 117]. However, according to our current knowledge only the orthogonal arrangement (90° angle) of the needle seems to permit a comparable degree of sensitivity to that achieved by the linear arrangement while simultaneously enhancing the robustness of the system.

As already mentioned, a stable electrospray is created at LC flow rates of 0.5 to 5 μL/min; but the typical flow rates of narrow-bore or analytical columns currently

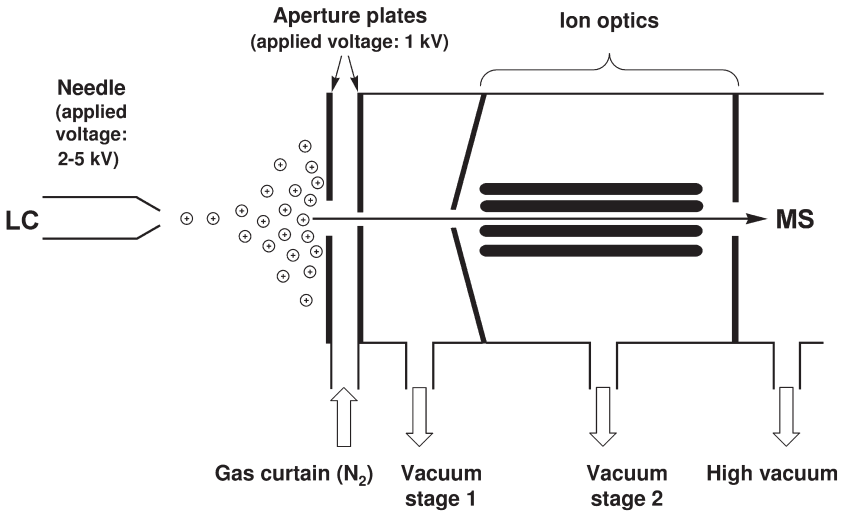


**Fig. 4.** Schematic set-up of an electrospray interface ("curtain gas" type described in [5])

used in routine analysis are higher by factors of 10 to 200. Therefore aerosol formation is optimised in the commercially available electrospray interfaces in order to establish the necessary compatibility. This can be achieved by the use of a nebuliser gas coaxially introduced into the needle ("ionspray": flow rate $<1.0$ mL/min [118]), by ultrasound ("ultraspray": flow rate $<0.5$ mL/min [119]) or by heat ("turbospray": flow rate $>0.1$ mL/min [120]). As discussed in Sections 3.1.2 and 3.1.3 the use of miniaturised LC/MS systems in routine analysis seems to offer a promising option for the future, even for analyses in biological materials. Special electrospray interfaces for low flow rates ($<200$ nL/min to 4 µL/min), known as "micro ESI" and "nanospray", that are currently still undergoing development and testing will play an important role in these advances [8, 20].

### 3.2.2.2 Atmospheric pressure chemical ionisation (APCI)

Modern atmospheric pressure chemical ionisation (APCI) sources are based on the ground-breaking developments pioneered by Horning et al. in the mid-1970s [121, 122]. Figure 5 shows the schematic set-up of a currently used APCI interface ("tube" type).

The liquid components of the eluent and the compounds dissolved in it are suddenly but still gently evaporated with the aid of a nebuliser and auxiliary gas introduced into the LC eluent through a coaxial capillary and using heat (300 to 500 °C) [5, 117]. Corona discharges from a discharge needle under high voltage (5 to 10 kV) generate free electrons. Alternatively, a $^{63}$Ni beta source provides electrons. The emitted electrons can interact with the gases present in the ionisation zone, such as

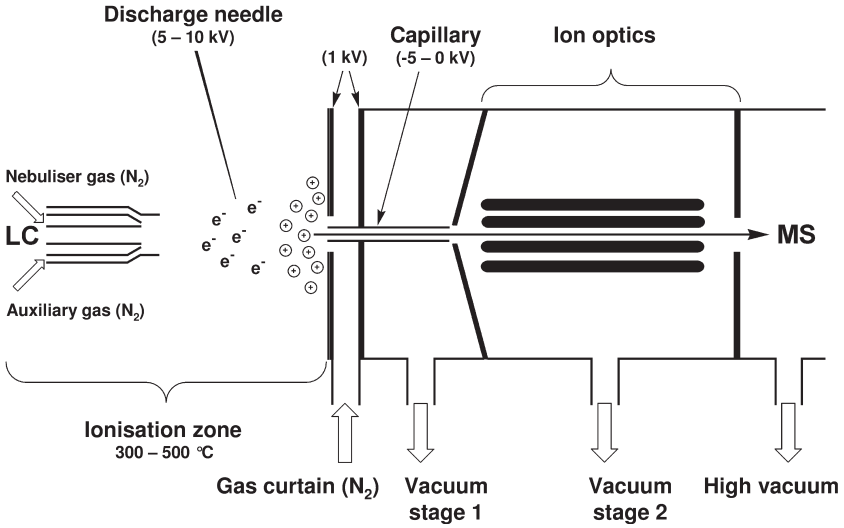


**Fig. 5.** Schematic set-up of an APCI interface ("tube" type as described in [5, 117])

nitrogen ($N_2$), oxygen ($O_2$), nitrogen monoxide (NO) and water ($H_2O$). This process creates a plasma that is rich in various reactant ions, the most important component being the solvated hydronium ion $H_3O^+(H_20)_n$ (Figure 6).

In the positive ion mode the reactant ions in the plasma, especially the solvated hydronium ion $H_3O^+(H_20)_n$, transfer protons or positive charges to neutral analyte molecules in the gaseous phase, whereas in the negative ion mode ionisation is achieved by proton removal or electron capture [124]. The proton affinity of water vapour (165 kcal/mol) decisively influences whether ionisation of the analyte molecule in the gaseous phase takes place or not, as solvated hydronium ions $H_3O^+(H_20)_n$ make up the main proportion of the reactant ions in the positive ion mode [125–127]. Only compounds with a higher proton affinity than water vapour become and remain protonated.

A detailed description of the mechanisms of chemical ionisation together with lists of the proton affinities of various compounds are found in the General Introduction to Volume 10.

The APCI ionisation process is extremely efficient and under ideal conditions the ion yield can be 100%. However, it should be noted that the analyte molecules compete with the matrix components of the sample and with the solvent molecules of the eluent for the reactant ions in the plasma. This can considerably influence the detection sensitivity of the relevant analyte(s) [5, 125–127]. The mass spectra that result from APCI generally exhibit a very prominent molecular ion of the compound to be detected in addition to a few fragment ions [122].

The ions formed are transmitted through a cone or a heated glass or metal capillary into the mass spectrometer in the commercially available instruments. As in ESI, the nebuliser has a linear or orthogonal arrangement with respect to the inlet to the mass

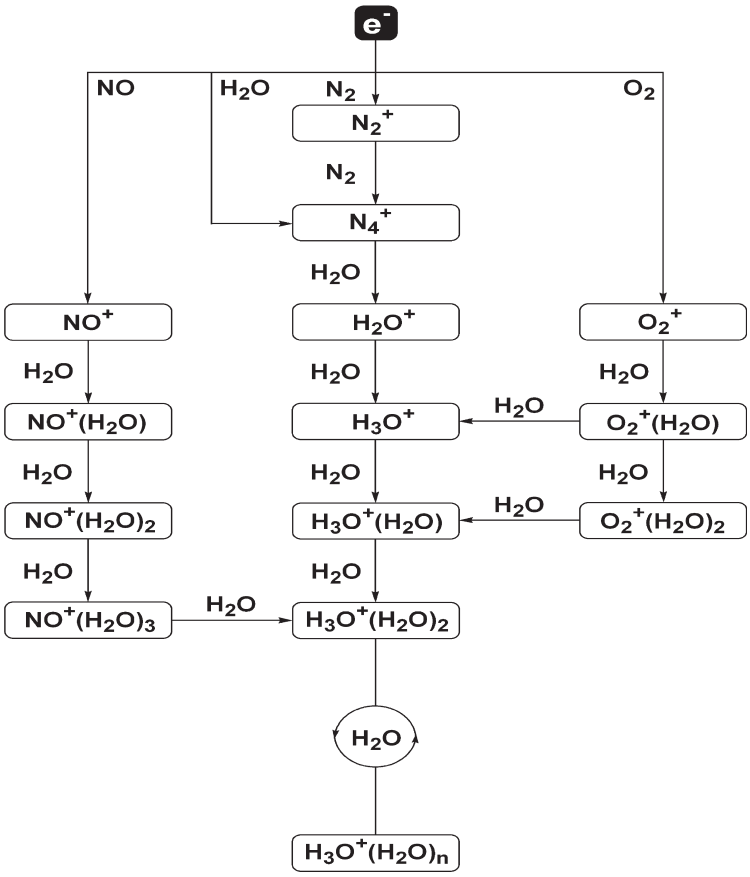


**Fig. 6.** The formation of the solvated hydronium ion $H_3O^+(H_20)_n$ at the APCI interface according to [5, 123]

spectrometer; in this case too, desolvation of the ions formed by interaction with the plasma ions is often achieved with the help of a counter-stream of hot nitrogen. The ion optics used (skimmer, octopole, lenses) and the arrangement of several vacuum chambers in series to attain the required high vacuum are identical to the technology used in ESI. This permits manufacturers to offer users LC/MS instruments in which an ESI source can be exchanged for an APCI interface, depending on the desired application. In such cases the same basic instrument can be equipped to perform analysis in both ionisation modes.

The typical LC flow rates that are compatible with APCI sources are between 0.5 and 2.0 mL/min. Such sources are thus ideal for use with analytical columns [5].

APCI has proved successful for the detection of ionisable polar and non-polar molecules with a molecular weight $<2000$ u or in cases where the LC eluents are relatively non-polar solvents [5, 6]. In particular, it is widely applied in the pharmaceutical

industry. APCI is an excellent complement to the analytical range accessible to ESI, which does not cover the above-mentioned applications. In other cases, namely the determination of biopolymers, APCI is inferior to ESI. For the above reasons and, as the technical changeover is simply performed, the complementary use of both ionisation techniques brings important advantages to many analytical laboratories.

### 3.2.2.3 Atmospheric pressure photo-ionisation (APPI)

Atmospheric pressure photo-ionisation (APPI) is a very new ionisation method for LC/MS. Robb et al. reported the first APPI source in 2000 [128]. Starting with a commercial APCI interface, they replaced the discharge needle by a vacuum ultraviolet lamp that emitted 10 eV photons. As in the case of the APCI source, the liquid eluent containing the dissolved compounds is evaporated suddenly but gently by a combination of a nebuliser and auxiliary gas (introduced coaxially with respect to the LC eluent) and by heat (450 °C) before the generated gases are irradiated by photons in the immediately adjacent photo-ionisation zone. There the following reaction can proceed in the positive ion mode:

$$M + h\nu \longrightarrow M^{\bullet +} + e^{-}$$

This involves the absorption of a photon by a molecule in the gaseous phase, the release of an electron and the formation of a positively charged molecule radical ion. This always occurs when the incident photon energy is greater than the ionisation energy of the molecule. Especially when protic solvents, elevated source pressure and prolonged photon irradiation are used, the protonated molecular ion is also found in addition to the dominant molecule radical ion [129].
Despite this, there is a low statistical probability that an analyte molecule will be ionised. To enhance the yield of analyte ions a suitable, readily photo-ionisable substance (dopant) can be introduced into the stream of auxiliary gas through a T-junction. After being initially photo-ionised, the dopant transfers its charge directly to the analyte molecule (reaction 1) if the electron affinity of the analyte is greater than that of the dopant. However, if the proton affinity of the analyte molecule is greater than that of the deprotonated dopant, solvent molecules and their clusters serve as intermediates for proton transfer from the dopant ion to the analyte molecule (reaction 2) [103, 130]:

$$D + h\nu \longrightarrow D^{\bullet +} + e^{-}$$
$$1)\ D^{\bullet +} + A \longrightarrow D + A^{\bullet +}$$
$$2)\ D^{\bullet +} + L \longrightarrow [D - H]^{\bullet} + LH^{+}$$
$$LH^{+} + A \longrightarrow L + AH^{+}$$

Anisole, benzene, toluene and acetone are commonly used dopants; the possible use of phenol is being investigated at present [103]. Acetonitrile represents a special case

among the LC solvents, as its solvent clusters can be directly photo-ionised and they can subsequently ionise analyte molecules [131, 132].
In the negative ion mode mainly deprotonated analyte ions (accompanied by adducts with solvent molecules and fragmentation reactions in the source) are formed from acidic analyte molecules in the gaseous phase by means of electron capture, dissociative electron capture or ion-molecule reactions, or analyte anions (accompanied by substitution products) are generated from analyte molecules with a positive electron affinity [103] (see General Introduction to Volume 10).
To date two commercial suppliers offer instruments with APPI interfaces. As in the case of ESI and APCI one manufacturer prefers a linear, the other an orthogonal arrangement of the nebuliser with respect to the inlet to the mass spectrometer. The set-up of the mass spectrometer and the inlet is identical to the known elements of instruments with other API sources.
In numerous applications narrow-bore columns are recommended and lower flow rates (e.g. 0.2 mL/min) than those recommended for the APCI source are suggested for the coupling of APPI interfaces with LC.
The spectrum of the substances that can be detected with APPI is largely identical to that already described for APCI on account of the great similarity in the ionisation processes. However, it has been tentatively concluded that detection limits lower than those yielded by other API sources may be achieved for more labile compounds, such as steroids, quinones or amino and nitro aromatic substances, due to the milder conditions in the APPI interface; in particular compared with APCI [103, 133, 134]. It remains to be seen whether this trend will be confirmed in the future; if this is indeed the case the value of APPI as an ionisation method will be further enhanced.

## 3.3 Mass filters

One or several mass filters can be coupled with the LC/MS interface. These filters separate the molecular ions according to their mass-to-charge ratios. The physicochemical principles of mass filters are comprehensively described in the General Introduction to Volume 10.

### 3.3.1 Mass filters in general

In general, the mass filters used for GC/MS are also widely employed in liquid chromatography/mass spectrometry. These include magnetic sector field [135], quadrupole [136] and ion trap instruments [137]. The individual instruments and their advantages and drawbacks have been comprehensively presented in the "General Introduction" to Volume 10. In addition, this chapter includes a detailed discussion of the principles of tandem mass spectrometry (MS/MS) using the triple quadrupole mass spectrometer and the ion trap as examples. Therefore we will discuss only mass filters that are used in LC/MS and mass filter combinations and that were not pre-

viously presented in the DFG "Analyses of Hazardous Substances in Biological Materials" collection of methods below.

### 3.3.2 Special mass filters

Additional mass filters that are employed in LC/MS are the time-of-flight (TOF) mass spectrometer, Fourier transform mass spectrometer (FT-MS) and the orbitrap. TOF instruments, especially in combination with other mass filters, are now well established in routine analysis; in contrast, the FT mass spectrometer and the orbitrap are mainly used in basic analytical research.

#### 3.3.2.1 Time-of-flight mass spectrometer

Time-of-flight mass spectrometers (TOF-MS) are based on a suggestion put forward by Stephens in the 1940s [138] and studies by Wiley and McLaren [139] in the 1950s. Figure 7 shows the principle of the time-of-flight mass spectrometer.
Molecular ions leave the ion source (e.g. an API source) and reach a zone where they are accelerated by an electric field. Then they travel a defined distance in the flight tube at a speed that depends on their mass before reaching the detector where their arrival is recorded. The time between the application of the acceleration impulse to the ions and their arrival is measured. The following equation applies:

$$t = \left(\frac{2\,m\,d}{e\,E}\right)^{1/2} + L\left(\frac{m}{2\,e\,V_0}\right)^{1/2}$$

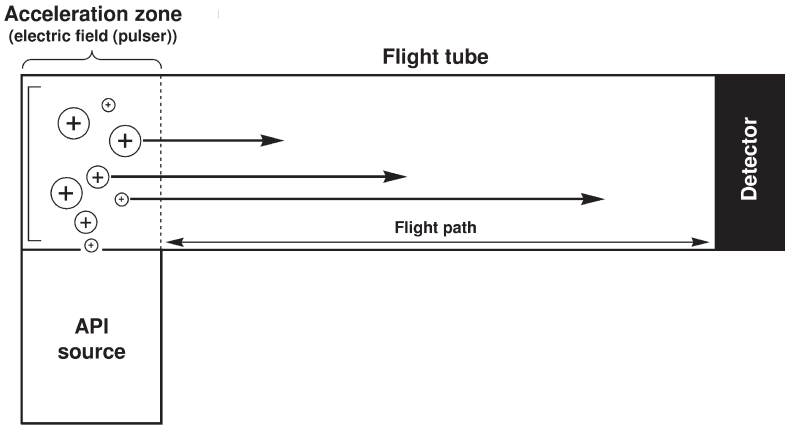


**Fig. 7.** Principle of the time-of-flight mass spectrometer (modified according to [5])

whereby $t$ designates the flight time, m the molecular mass, $d$ the length of the acceleration zone, e the ion charge, $E$ the strength of the applied electrostatic field, $L$ the length of the flight path without a field and $V_0$ the applied acceleration voltage. Typical flight times of molecular ions range from 5 to 100 microseconds [140].

Whereas general mass filters record the spatial separation of the ions to yield mass spectra, temporal separation of the ions in the TOF mass spectrometer has two decisive advantages: the (theoretically) unlimited mass range and the extremely rapid and sensitive recording of complete mass spectra [5, 141–143]. In this case all the incoming ions are simultaneously registered, so the time-consuming scanning of a previously defined mass range becomes unnecessary. Thus $>10^6$ u/s can be recorded compared with 4000 u/s for quadrupole and ion trap instruments [5]. However "selected ion monitoring" (SIM) for further enhancement of sensitivity is impossible due to instrumental design.

One disadvantage of a linear time-of-flight mass spectrometer (Figure 7) is its low resolution power R = 1000 [140]. This is due to the difference in the time the ions of identical mass required for formation in the source region, to their different spatial arrangement in the electric field of the acceleration zone, and to the variation in the initial kinetic energy of the ions before they receive the acceleration impulse in the direction of the flight tube [5]. Technical improvements in the acceleration of the ions [139], and in particular the introduction of the reflectron by Mamyrin et al. [144], have considerably increased the resolution (R = 8000 to 10 000 [141]) in modern time-of-flight mass spectrometers (Figure 8).

The electric fields of the reflectron reduce the speed of the ions and reflect them in the direction of the detector. If ions with the same mass-to-charge ratio but with different kinetic energy reach the reflectron, then the ions with the higher energy (and

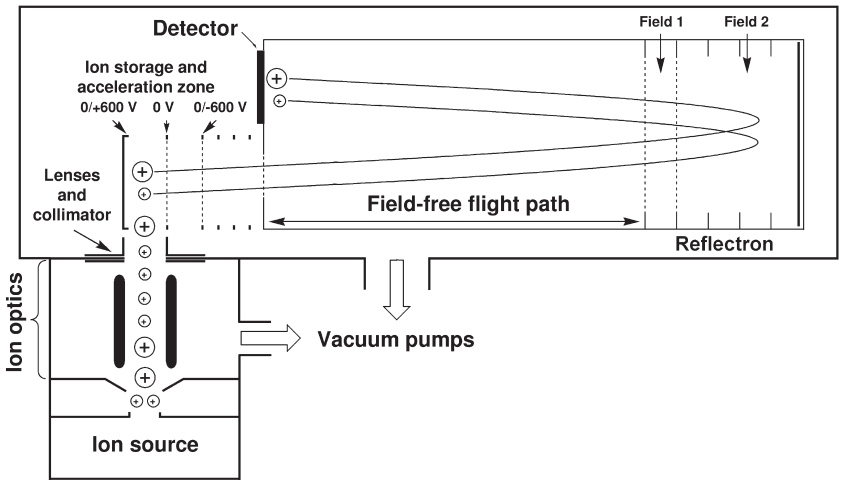


**Fig. 8.** Schematic set-up of a modern time-of-flight mass spectrometer with orthogonal injection and reflectron (modified according to [142])

velocity) penetrate deeper into the electric fields of the reflectron than the ions with lower energy (and velocity). Thus the former spend more time in the reflectron and their velocity is adjusted to that of the slower ions with the same mass-to-charge ratio on their further flight path. This ensures that all the ions with the same mass-to-charge ratio reach the detector simultaneously.

The coupling of an API source to a mass spectrometer that is typical for modern LC/MS proves more difficult in the case of the TOF mass spectrometer than for general mass filters (Section 3.3.1). The reason is that API sources, such as the ESI interface, generate a continuous ion beam that can be readily detected by continually operating mass spectrometers such as quadrupole spectrometers. In contrast, discontinuous acceleration impulses applied to the ions are absolutely necessary for measurements in TOF mass spectrometry. An orthogonal arrangement of the API source with respect to the TOF mass spectrometer creates an ion storage and acceleration zone from which discrete "packets" of the ion beam that is being continuously generated by the API source can be accelerated into the flight tube (Figure 8). Ion optics installed before the flight tube permit the velocity of the ions to be minimised by collisional damping and cause focusing of the ion beam, which leads to heightened sensitivity and resolution [141, 143]. In this way complete mass spectra of the eluted components can be obtained, even from narrow peaks (elution time: 0.1 to 1 s) like those generated by micro-bore columns [143].

The TOF mass spectrometer has proved successful with LC/MS coupling on account of its (theoretically) unlimited mass range and the extremely rapid and sensitive recording of the complete mass spectra of peptides and especially of proteins with high molecular weights. In tandem mass spectrometry it often replaces the third quadrupole and allows the highly sensitive recording of complete mass spectra of the daughter ions (Section 3.3.3).

#### 3.3.2.2 Fourier transform mass spectrometer

The fundamental principle behind Fourier transform mass spectrometers (FT mass spectrometers) or ion cyclotron resonance mass spectrometers (ICR mass spectrometers) was described in the 1930s by E.O. Lawrence [145], and it was first applied in mass spectrometers by Marshall et al. in 1974 [146].

FT mass spectrometers are also known as "magnetic ion traps". The ions, e.g. from an API source, reach the trap where they oscillate around the axis of a very strong magnetic field that is applied. Their frequency of rotation is inversely proportional to their mass. The rotation of the ions induces an electrical current from which their frequency of rotation can be determined. The measured frequency of rotation is converted to mass by Fourier transformation [5].

The advantages of FT mass spectrometry are the large mass range (>15 000 u) that can be measured and the extremely high resolution (<500 000). Moreover, in contrast to all other mass filters, the ions are not destroyed during detection, which permits highly sensitive determination of product ions following multiple dissociation reactions ($MS^n$ experiments). One great drawback is the high cost involved in purchasing

and maintaining the instruments. Therefore at present FT mass spectrometers are mainly used in basic analytical research for structural clarification of compounds. In a recent review [147] the author points out that numerous commercial suppliers are currently endeavouring to introduce "benchtop" FT mass spectrometers onto the market. It remains to be seen whether these instruments will also provide a feasible option in the near future for laboratories performing routine analysis where they may be coupled with LC mainly for structural clarification.

#### 3.3.2.3 Orbitrap

The most recent development of the ion trap mass spectrometer is the "orbitrap" [148]. This ion trap contains a central, spindle-shaped electrode. The ions are injected into the orbitrap tangentially with respect to this electrode, and they orbit around the central electrode as a result of electrostatic attraction. At the same time they oscillate back and forth along the axis of the central electrode. The frequency of these oscillations generates electric signals at the detector plate, which are converted to the corresponding m/z ratios by Fourier transformation. The principle is therefore similar to ICR-MS, but it functions with an electrostatic field instead of a magnetic field. For this reason orbitraps do not require a complex cooling system with liquid helium, as no supraconducting magnetic field has to be generated. The mass resolution power of orbitraps is only slightly poorer than an ICR-MS instrument with a 7-Tesla magnet. We refer interested readers to the primary literature for more detailed information [149–151]. As these instruments are considerably less expensive to maintain than FT mass spectrometers, orbitraps coupled with LC will certainly be useful for both structural clarification of proteins and for the detection of "smaller" molecules in the future.

### 3.3.3 Combinations

Tandem mass spectrometry has already been introduced in Volume 10 with the triple quadrupole and ion trap serving as examples. The significance of different scanning techniques in LC/MS will be briefly discussed below using well-known examples.
Triple quadrupole instruments are equipped with various scanning techniques that can be applied individually or in combination. The "selected reaction monitoring" (SRM) or the "multiple reaction monitoring" (MRM) mode is especially suitable for the necessary sensitive and exact quantification of analyses in biological materials. A characteristic precursor ion is selected in a SIM (selected ion monitoring) measurement procedure in the first quadrupole (Q1). Then it is accelerated against a collision gas by a certain voltage in the second quadrupole (Q2), which functions as a collision cell, and the resulting product ion is selectively detected in the third quadrupole (Q3). Such SRM or MRM transitions are extremely characteristic and are accompanied by a low background signal. Therefore it is possible to achieve very specific and sensitive detection of a compound.

The electronic parameters can be rapidly changed in the quadrupoles and the collision cell can be quickly evacuated again due to the special quadrupole geometry. In this way contamination due to successive analytes (known as "cross talk") can be prevented and a large number of target ions can be detected simultaneously and quantitatively evaluated.
A further scanning technique is the "product ion" scan that can yield important information for structural clarification. Like the MRM technique, it is based on the collision of precursor ions with collision gases.
A function that also serves for structural clarification is the "neutral loss" scan, which is based on the principle of fragmentation of a precursor ion. However, in this case the cleavage of a neutral fragment that cannot be detected directly is measured indirectly. This technique is important for the determination of glucuronides, sulphates or mercapturic acids, which cleaved as neutral particles from the xenobiotic parts. Thus these metabolites can be readily identified and quantified in complex matrices. Measurement is based on the principle that Q1 and Q3 carry out scans staggered by the difference in the mass of the neutral particle, and only those mass transitions that are exactly equivalent to this difference in mass are detected, e.g. m/z = 176 u in the case of a glucuronide.
The "precursor ion" scan is the 4th mode that may be important for structural analysis. In this case a product ion is permanently measured in Q3, while Q1 simultaneously scans a pre-defined mass range. Whenever the product ion is detectable, it is accurately assigned to a precursor ion and this information can be used for structural clarification. For example, modified DNA bases can be found in this way, as unmodified bases form a stable product ion as a rule.
An MS/MS coupling known as "tandem in space" is required for the "neutral loss" and "precursor ion" scanning modes, as the MS/MS analysis steps must be spatially separated (Q1, Q2 = collision cell, Q3), e.g. in the triple quadrupole instrument. A "product ion" scan can also be performed by an ion trap.
In recent years further combinations have been introduced by commercial suppliers for use in routine analysis to complement the conventional tandem mass spectrometers. These include the triple quadrupole with the linear ion trap (Qtrap) and a combination of two quadrupoles with a TOF mass spectrometer (QqTOF).

#### 3.3.3.1 Combination of a triple quadrupole and an ion trap (Qtrap)

Qtrap technology is a combination of a tandem mass spectrometer (triple quadrupole mass spectrometer) and a linear ion trap, whereby the second analytical quadrupole (Q3) can serve as a quadrupole or as a linear ion trap. Such a mass spectrometer incorporates the technology to perform all the types of scans that are possible using a traditional tandem mass spectrometer and can also be operated with the second analytical quadrupole acting as a linear ion trap, e.g. to perform $MS^n$ experiments.
However, "in space" fragmentation, typical for tandem mass spectrometers, is carried out in the $MS^2$, whereas the $MS^3$, like the ion trap, operates on the principle of "in time" fragmentation. "In space" fragmentation as a result of collisions of the selected precursor ions with inert gas molecules is more effective than "in time" fragmenta-

tion in ion traps. For this reason the software of such combinations usually restricts the $MS^n$ experiments to $MS^3$. Besides more effective cleavage, "in space" fragmentation has the additional advantage that all fragments are detected, which is physically impossible in an ion trap. The product ion spectra thus obtained show the typical fragmentation patterns of a tandem mass spectrometer.
This instrument offers several additional advantages for analyses in biological materials. Compared with traditional tandem mass spectrometers or ion traps considerably more sensitive product ion spectra can be recorded in the "enhanced product ion" (EPI) mode. This offers the possibility of identifying unknown substances by the acquisition of complete product ion spectra. In addition, these fragments can be fragmented again and analysed by $MS^n$ for further characterisation.
An intelligent software solution, known as "information-dependent acquisition", allows different types of scanning to be freely combined during the same analysis, e.g. a "constant neutral loss" (CNL) scan followed by an EPI scan. For instance, glucuronic acids result in a CNL of $m/z = 176$ u. If this CNL is combined with an EPI, a corresponding EPI spectrum is recorded for each detected CNL signal. For example, intensive CNL signals alone may be selected by means of freely adjustable parameters, then a mass spectrum can be recorded with EPI, or mass spectra with different collision energies can also be generated to provide additional information for characterisation. This coupling permits more information to be acquired in a shorter time with comparable sensitivity. There is no necessity for multiple measurements.
The combination of an MRM scan with an EPI scan yields very high sensitivity and highly reliable identification, as the corresponding product ion spectrum is available for each MRM signal.
Thus, for example, the combination of an MRM with an EPI scan improves the reliability of identification in the detection of glucuronides by means of MRM, in which the transition of $M^{+/-}$ to [$M^{+/-}$ -176 u] is the most sensitive transition in many cases. In this case a complete mass spectrum replaces an additional qualifier transition [24]. As urine contains a large number of glucuronides, so that other isobar glucuronides with this characteristic transition are observed as well as the target analyte, this combination of scans permits reliable detection of the glucuronide in question.
Reviews on the application of the Qtrap technique are to be found in [152–154].

#### 3.3.3.2 Combination of quadrupole and TOF (QqTOF)

QqTOF represents a further hybrid instrument that integrates a TOF mass spectrometer for detection instead of the second analytical quadrupole. In addition to ion traps, QqTOF instruments are frequently used in the mass spectrometric investigation of proteins to characterise peptides. In the "small" molecule range it is suitable for characterising unknown analytes and metabolites due to its high mass accuracy of $< 10$ ppm. The high mass accuracy of the QqTOF instruments permits exact determination of the empirical formulae of unknown analytes. The structural formula can be established with the aid of appropriate software and of databases. Therefore laboratories in the chemical industry investigating metabolism are the main users, as QqTOF in-

struments enable structural clarification of unknown metabolites of new active substances in the pharmaceutical and pesticide sectors. Comparison of QqTOF instruments with other LC/MS devices and a comprehensive discussion on the advantages and disadvantages of this technique are found in [155–157].

### 3.4 Detectors

The detectors commonly employed in LC/MS (secondary electron multiplier or photon multiplier) are the same as those used for GC/MS. A detailed description of their function is given in the General Introduction to Volume 10.

## 4 Aspects of the development and optimisation of methods

### 4.1 General aspects

As described in detail in Section 3.2, the ionisation process in LC/MS takes place at atmospheric pressure using the normal ESI, APCI and APPI sources. Figure 9 shows the application ranges of each type of source.

The criteria for the selection of a suitable mass spectrometer or a combination of mass spectrometers are presented in the General Introduction to Volume 10 (sector field device, quadrupole instrument, ion trap) or in Section 3.3 (TOF mass spectrometer, FT mass spectrometer, orbitrap) of this chapter.

The following substance groups are possible candidates for detection in biological materials with the aid of LC/MS. All the metabolites of phase II metabolism, such as sulphates, glucuronides, acetates, amino acid conjugates and the mercapturic acids

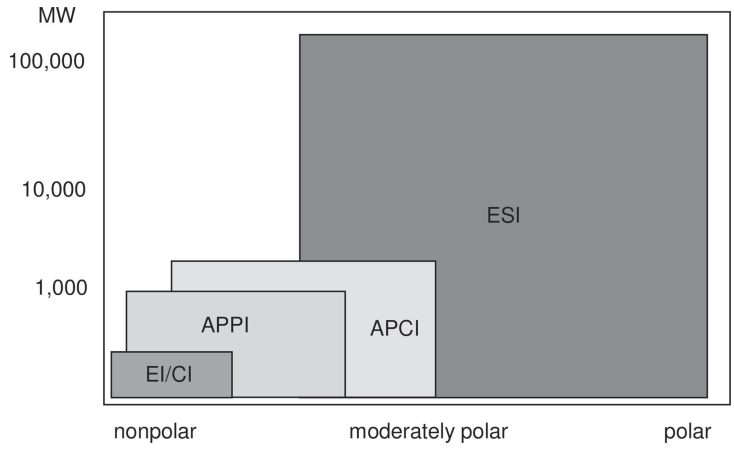


**Fig. 9.** Operational ranges of the individual ion sources

from glutathione-*S* conjugates are basically suitable for detection and quantification by means of LC/MS. In many cases DNA adducts, organic acids and compounds containing nitrogen can also be sensitively analysed as biomarkers by LC/MS. Conversely, aldehydes, ketones or very non-polar substances are rather unsuitable. However, methods have been described for LC/MS analysis of aldehydes, but they require prior derivatisation [158, 159]. Table 5 contains a selection of current LC/MS methods for the assay of xenobiotics in biological materials. As several hundred articles describing LC/MS applications were published in 2006 alone, this table can only present a selective overview. It specifies the substance itself and the metabolite class to which it belongs, as biological monitoring uses the metabolite as a biomarker in addition to the parent compound in some cases. As a rule the xenobiotics/metabolites are hydrophilic analytes that are suitable for detection by HPLC and that can be analysed with widely used ionisation techniques such as ESI and/or APCI. Thus, for example, bisphenol A can be very sensitively determined both with the electrospray [24] and with APCI ionisation techniques [160]. Blood, plasma, serum and urine samples are generally analysed in the field of biological monitoring. In some cases samples of human milk are investigated to detect xenobiotics. Independent of these different sample matrices, the same LC/MS method can be applied after appropriate sample processing. In some cases protein precipitation may be the only preparation step necessary for all matrices, e.g. for urine and human milk [160, 161].

It is no simple matter to transfer methods from one laboratory to another or from one LC/MS instrument to another on account of the different ionisation techniques (especially ESI and APCI) and due to differences in the individual interfaces from different manufacturers. In contrast to GC/MS, generally applicable spectra libraries are not available. They are only valid for the manufacturer concerned and generally only for a specific type of instrument. Nevertheless, the molecular ions and mass fragments of an analyte generated by different instruments are comparable, but they often differ in their intensities or their ratios. This also applies to molecular ions, as M+X cluster ions may also be generated, depending on the interface and ionisation parameters. Further information must therefore be taken from the primary literature, as many parameters that cannot be discussed in detail here have to be adjusted to adapt a method.

In addition to calibration of the instrument with appropriate standard substances (reserpine, polypropylene glycol mixtures) that are generally specified by the manufacturer, the entire process of ionisation, ion separation and detection is carried out specifically for the relevant analyte. This fine adjustment is known as “tuning”. This is decisively different to the procedure for GC/MS, as the calibration and tuning are usually performed with a tuning substance independent of the analyte. Therefore tuning is a relatively time-consuming process in LC/MS analysis, especially when several compounds are to be analysed, but it is absolutely essential in order to achieve sensitive measurements.

The influence of the LC eluent and its composition as well as the influence of buffer solutions or the addition of acid on the ionisation process can be tested and then optimised to obtain the highest sensitivity. If available, different ion sources can be compared with regard to the highest attainable ion yield for the relevant substance.

**Table 5.** Examples of LC/MS methods to detect xenobiotics in biological materials

| Xenobiotic | References |
|---|---|
| **Mercapturic acids** | |
| Acrylamide | [34, 162–164] |
| Benzene | [165] |
| 1-Bromopropane | [166] |
| 1,3-Butadiene | [167] |
| Styrene | [168] |
| Vinyl chloride | [169, 170] |
| Xylene | [171] |
| **Glucuronides** | |
| Aromatic amines | [172] |
| Bisphenol A | [24, 160, 173] |
| 1-Bromopropane | [166] |
| Daidzein | [174] |
| Ethanol | [71, 175–177] |
| n-Hexane | [178, 179] |
| Nicotine | [180] |
| PAH | [181] |
| Pesticides | [182] |
| Phthalates | [43, 183–187] |
| Tetrabromobisphenol A | [188] |
| **Sulphates** | |
| 1-Bromopropane | [166] |
| Ethanol | [71, 175–177] |
| Pesticides | [182] |
| PAH | [181] |
| **Organic acids** | |
| Benzene | [165] |
| Methyl parathion | [189] |
| Organophosphates | [190] |
| Trichloroethene and tetrachloroethene | [191] |
| Parabens | [192, 193] |
| Perfluorinated surfactants | [194–196] |
| Phthalates | [184, 197–199] |
| **Aromatic amines** | |
| Aminobiphenyl derivatives | [200] |
| Heterocyclic aromatic amines | [201, 202] |
| Nitro aromatic compounds | [133, 134] |
| **DNA adducts** | |
| Dimethylnitrosoamine | [33] |
| Oxidative stress | [33, 203] |

As a rule, quantitative analyses are carried out at LC flow rates in the range of 150 to 400 µL/min (in the case of APCI up to 1 mL/min). This leads to an ionisation yield that is far less constant than that achieved by GC/MS. Ionisation is also affected by impurities in the LC eluent and in the inert gases used to assist evaporation of the eluent as well as contamination of the source by matrix components that are

not removed by the vacuum system at atmospheric pressure. Although reduced sample preparation and coupling with an autosampler permit several hundred analyses to be performed per day, thus saving time and lowering the cost of analysis, serious contamination of the ion source results when complex biological matrices such as blood and urine are analysed. Compared with past generations, however, the latest instruments are comparatively robust under these adverse conditions.

In general, the use of internal standards for quantitative analysis by means of LC/MS considerably increases the accuracy of the method. Analytes labelled with isotopes ($^{2}H$, $^{13}C$, $^{15}N$ or $^{18}O$) are to be recommended as internal standards insofar as they are available on the market or can be readily synthesised. As the chemical and physical behaviour of these compounds differs only minimally from that of the actual analyte, they are eminently suitable for the compensation of errors or interferences (e.g. during sample preparation or in the ion source).

## 4.2 Development and optimisation of methods

Figure 10 shows the general procedure to be followed for the development and optimisation of an LC/MS method.

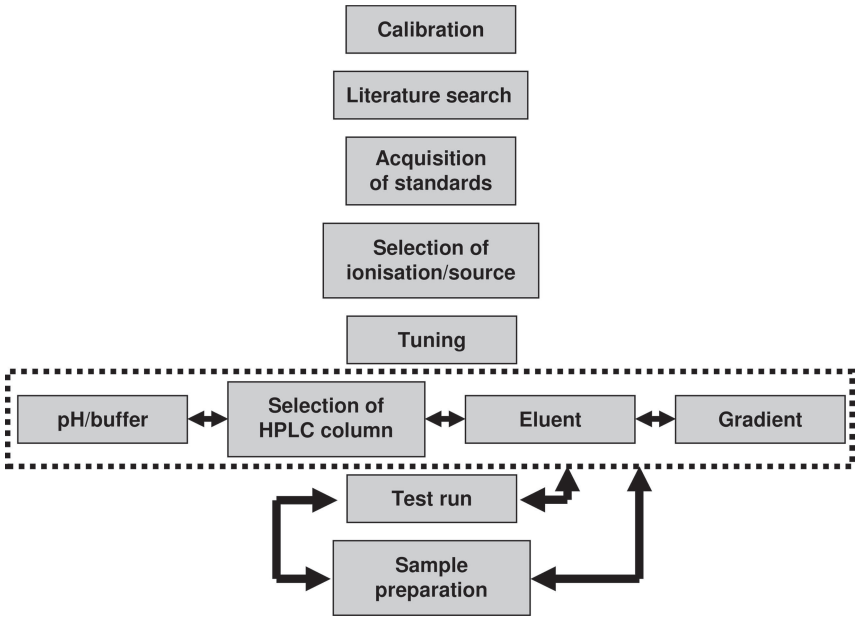


**Fig. 10.** The individual steps to be followed for the development and optimisation of an LC/MS analytical method

## Calibration

The instrument should be initially calibrated with the standard substances (e.g. polyethylene glycol standard) specified by the relevant manufacturer in order to ensure that the exact mass-to-charge ratio m/z is obtained during tuning. The procedure to be followed is given in the instructions provided by the commercial supplier of the mass spectrometer.

## Literature search

A search for relevant publications should be the first step in method development in order to save time. Important information is obtained when a search targets the ionisation technique (ESI, APCI, APPI), the charge (positive, negative), the LC conditions used (column, capillary, buffer, organic solvent, gradient, flow rate) for the substance or class of substance concerned, e.g. under "Materials and Methods" in publications or in collections of methods such as the one presented here. Data on the detection limit and the validity criteria provide additional indications of the applicability and the reliability of the method. In rare cases, for example, if the method is described for the same analytical equipment (LC and MS system) and the given detection limit is satisfactory for the desired application, the method can be taken over in its entirety and tuning becomes unnecessary.

## Tuning

If no published data are available, the structural formula of the analyte provides the first important indications. Lipophilic substances, such as steroids, nitro aromatic compounds, etc., are more readily ionised by APPI or APCI than by ESI. It should also be considered whether the analyses are not easier to perform by means of GC/MS. In contrast, hydrophilic analytes, such as all phase II metabolites, are generally very suitable for the ESI technique. Substances with acidic protons (organic acids, phenols, etc.) are more readily analysed in the negative ionisation mode, whereas compounds that can easily accept a proton (many compounds containing nitrogen) are better detected using the positive ionisation mode.
Numerous standard substances are commercially available today. However, some substances (e.g. certain phase II metabolites) have to be synthesised in-house or by a contract laboratory. The glucuronides represent a special group of standards, as their synthesis proves very laborious. However, they can be isolated in small quantities by microsomal incubation or from the urine of experimental animals or from human urine [204, 205]. As no weighable amounts are generally obtained in this way, the standard solution of glucuronide can be quantified either by UV spectroscopy or following enzymatic cleavage of the starting substance. Calibration solutions can then be prepared from this solution [206].

In the first experimental step the analyte or the standard substance is dissolved in the solvents normally used for LC/MS (water, acetonitrile, methanol) and dilutions in the concentration range from 1 to 100 ng/µL are prepared. If the positive ionisation mode is to be used, the ion yield can be considerably enhanced by acidification of the solution to a pH of 1 using formic acid. In contrast, acidification tends to diminish the ion yield in the negative ionisation mode. Then the MS parameters for the substance under investigation are "tuned" with the aid of the syringe pump (Section 3.1.1). Depending on the type of instrument, flow rates ranging from 5 to 50 µL/min are necessary for this procedure when ESI is used. APCI and APPI often require higher flow rates, and APPI also requires the addition of dopants such as toluene (Section 3.2.2.3).
In addition, solvents that are filled into the syringe pump must be degassed and free from bubbles in order to achieve a stable spray and thus a stable signal.
Initially, the default gas flow and voltage settings of the relevant mass spectrometer are used in the "full scan" mode. The mass range must cover the molecular ion mass of the analyte. If the relevant $[M+H]^+$ or $[M-H]^-$ ion of the substance is detected with a signal-to-background noise ratio (S/N) of more than 10 under these conditions, then the optimisation process can be initiated by adjusting the instrumental voltages. Depending on the manufacturer, the relevant instrumental software may offer "autotuning" functions that save the user the task of manually optimising the different voltages. Such a function saves considerable time, particularly in the case of triple quadrupole instruments, as more than 5 different voltages must be optimised, against each other in some cases. More details are found in the appropriate user manuals.

### Integration/optimisation of the LC

The next step is to integrate the liquid chromatographic system, usually an HPLC system. The chromatographic behaviour of the analyte is influenced by the choice of a suitable HPLC separation column (Section 3.1.2) and HPLC solvents, by the use of buffer solutions (whereby the use of more volatile salts, such as ammonium formate is preferable to less volatile salts), and by suitable gradients. These parameters are selected to ensure that as high an S/N ratio as possible is attained at the highest possible proportion of organic solvent in the HPLC eluate and at low flow rates of 150 to 300 µL/min (in the case of ESI sources). More modern ESI sources operate at higher flow rates and are optimised for these flow rates. APCI and APPI sources also run at higher flow rates. We refer readers to the relevant user manuals.
An HPLC-UV detector connected in series can also assist this optimisation. A high proportion of organic solvent and slow flow rates result in a very "dry" electrospray, i.e. the eluent components can be evaporated very effectively and an optimum ion yield is achieved.
The final step is to optimise the gas flow rates under the established HPLC conditions and, if necessary, the voltages to be applied, especially in the ion source, to attain the best possible ion yield. In the case of a single quadrupole instrument the mass spectrometer is operated in the "selected ion monitoring" (SIM) mode. In con-

trast, tandem mass spectrometry (e.g. ion trap or triple quadrupole instrument) must be operated in the "selected reaction monitoring" (SRM) or "multiple reaction monitoring" (MRM) mode. Two procedures are followed:

**A**. No HPLC column is used, but the flow rate is set at the value that has been established for chromatographic separation. The solvent ratio of the HPLC eluent is that at which the analyte is eluted from the HPLC column. Identical quantities of an analyte standard mixture are injected under these conditions and the gas flow and instrumental voltages are adjusted until the highest signal-to-background noise ratio is obtained.

**B**. The HPLC column to be used can be connected or not, but the flow rate is set at the value that has been established for chromatographic separation. The HPLC solvent composition is that at which the analyte is eluted from the HPLC column. In this procedure the analyte standard mixture already used for tuning is mixed with the eluate from the HPLC column through a T-junction, e.g. by means of a syringe pump (Section 3.1.1) and at a flow rate of 5 to 50 µL/min. This procedure permits continual optimisation of the appropriate parameters (which saves time as a rule) and is advisable for the tuning process with an APCI or APPI ion source, as these operate at higher flow rates.

In both cases it is advisable to prepare the analyte standard mixture in the eluent mixture in which the analyte is eluted from the HPLC column. This prevents the occurrence of any double signals that may occur (after the liquid chromatographic system has been integrated) due to the fact that the analyte is not fully retained on the separation column as a result of an excessive proportion of organic solvent. If the organic proportion is too low, the analyte may not be completely dissolved in some cases.
The first analytical run can then be started with the complete system and all the parameters can be checked again. The process described above must be carried out anew for other analytes. A separate tuning process is not generally required in the case of isotope-labelled internal standards. However, when a triple quadrupole instrument is used, it may be necessary to check the fragmentation of the internal standard, especially if the product ions cannot be clearly assigned. If no isotope-labelled standards are available, a standard substance must be selected that most closely resembles the analyte to be determined with regard to its ionisation and chromatographic behaviour. The complete tuning process must be carried out to ensure that this condition is fulfilled.
The necessary indices for evaluation of the method (precision, accuracy, detection limit and interference) are determined on the basis of calibration solutions. If possible, these should be prepared in the matrix that will later become the sample matrix.

## 5 Perspectives

Liquid chromatography/mass spectrometry (LC/MS) combines the liquid chromatography (LC) and mass spectrometry (MS) analytical techniques. LC/MS instruments have been commercially available from various suppliers for the past two decades.

Most of the earlier publications on LC/MS coupling emanated from pharmacokinetic laboratories of the pharmaceutical industry. This technology has almost completely superseded GC/MS in this field, as hydrophilic metabolites of medicinal substances are ideal analytes for the LC-ESI-MS technique. Therefore it is not surprising that this technique is becoming increasingly established in biological monitoring. For instance, in the year 2000 there were 67 entries under the term "Monitoring AND LC/MS" in the "Pub-Med" database, in 2003 the number had grown to 133 and in 2006 it had reached 251.

In view of this rapid development it is not easy to speculate on the future of LC/MS. But various trends are recognisable, some of which have already been pointed out at appropriate points in the text and are briefly mentioned here again. Further miniaturisation of the systems in the field of liquid chromatography and accompanying enhancement of sensitivity at the detector are to be expected. This also applies to the development of LC/MS interfaces; miniaturisation is also probable in this case. Advances in sample preparation will minimise manual activities and progress towards complete automation (e.g. automated on-line SPE on special column material). The introduction of "benchtop" FT mass spectrometers and wider use of the orbitrap is foreseeable. At the same time this opens up opportunities for new mass spectrometer combinations. Therefore, with a view to biological monitoring, further improvement of detection sensitivity and specificity may be expected from future developments.

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