1
LC/MS Coupling

1.1
State of the Art in LC/MS

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1.1.1
Introduction

The dramatically increased demands on the qualitative and quantitative analysis of more complex samples are a huge challenge for modern instrumental analysis. For complex organic samples (e.g., body fluids, natural products, or environmental samples), only chromatographic or electrophoretic separations followed by mass spectrometric detection meet these requirements. However, at certain moments, a tendency can be observed in which a complex sample preparation and pre-separation is replaced by high-resolution mass spectrometer with atmospheric pressure (AP) ion sources. However, numerous ion – molecule reactions in the ion source – especially in complex samples due to incomplete separation – are possible because the ionization in typical AP ion sources is nonspecific [1]. Thus, this approach often leads to ion suppression and artifact formation in the ion source, particularly in electrospray ionization (ESI) [2].

Nevertheless, sources such as atmospheric-pressure solids-analysis probe (ASAP), direct analysis in real time (DART), and desorption electrospray ionization (DESI) can often be successfully used. In ASAP, a hot nitrogen flow from an ESI or AP chemical ionization (APCI) source is used as a source of energy for evaporation, and the only change to an APCI source is the installation of an insertion option to place the sample in the hot gas stream within the ion source [3]. This ion source allows a rapid analysis of volatile and semi-volatile compounds, and, for example, was used to analyze biological tissue [3], polymer additives [3], fungi and cells [4], and steroids [3, 5]. ASAP has much in common with DART [6] and DESI [7]. The DART ion source produces a gas stream containing long-lived electronically excited atoms that can interact with the sample and thus desorption and subsequent ionization of the sample by Penning ionization [8] or proton transfer from protonated water clusters [6] is realized. The DART source is used for the direct analysis of solid and liquid samples.

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A great advantage of this source is the possibility to analyze compounds on surfaces such as illegal substances on dollar bills or fungicides on wheat [9]. Unlike ASAP and DART, the great advantage of DESI is that the volatility of the analyte is not a prerequisite for a successful analysis (same as in the classic ESI). DESI is most sensitive for polar and basic compounds and less sensitive for analytes with a low polarity [10]. These useful ion sources have a common drawback. All or almost all substances in the sample are present at the same time in the gas phase during the ionization in the ion source. The analysis of complex samples can, therefore, lead to ion suppression and artifact formation in the AP ion source due to ion-molecule reactions on the way to the mass spectrometry (MS) inlet. For this reason, some ASAP applications are described in the literature with increasing temperature of the nitrogen gas [5, 11, 12]. DART analyses with different helium temperatures [13] or with a helium temperature gradient [14] have been described in order to achieve a partial separation of the sample due to the different vapor pressures of the analyte. Related with DART and ASAP, the direct-inlet sample APCI (DIP APCI) from Scientific Instruments Manufacturer GmbH (SIM) was described 2012, which uses a temperature-push rod for direct intake of solid and liquid samples with subsequent chemical ionization at AP [15]. Figure 1.1 shows a DIP-APCI analysis of a saffron sample (solid, spice) without sample preparation with the saffron-specific biomarkers isophorone and safranal. As a detector, an Agilent Technologies 6538 UHD Accurate-Mass Q-TOF was used. In the upper part of the figure, the total ion chromatogram (TIC) of the total analysis and in the lower part the mass spectrum at the time of 2.7 min are shown. The analysis was started at 40 °C and the sample was heated at 1°s^{-1} to a final temperature of 400 °C.

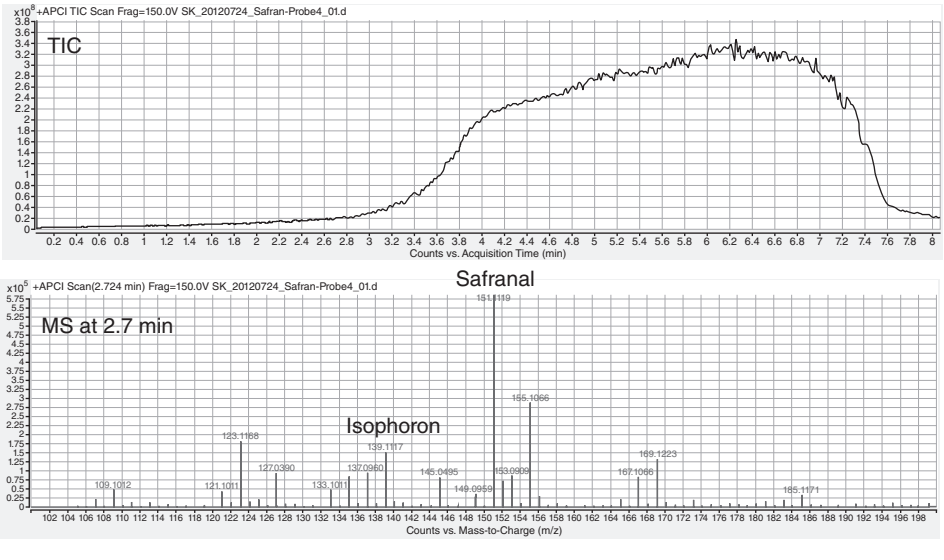

Figure 1.1 Analysis of saffron using DIP-APCI with high-resolution QTOF-MS.

These ion sources may be useful and time-saving but for the quantitative and qualitative analysis of complex samples a chromatographic or electrophoretic preseparation makes sense. In addition to the reduction of matrix effects, the comparison of the retention times allows also an analysis of isomers.

1.1.2 Ionization Methods at Atmospheric Pressure

In the last 10 years, several new ionization methods for AP mass spectrometers were developed. Some of these are only available in some working groups. Therefore, only four commercially available ion sources are presented in detail here. The most common atmospheric pressure ionization (API) is ESI, followed by APCI and atmospheric pressure photo ionization (APPI). A significantly lower significance shows the atmospheric pressure laser ionization (APLI). However, this ion source is well suited for the analysis of aromatic compounds, and, for example, the gold standard for polyaromatic hydrocarbon (PAH) analysis. This ranking reflects more or less the chemical properties of the analytes, which are determined with API MS:

Most analytes from the pharmaceutical and life sciences are polar or even ionic, and thus efficiently ionized by ESI (Figure 1.2). However, there is also a considerable interest in API techniques for efficient ionization of less or nonpolar compounds. For the ionization of such substances, ESI is less suitable.

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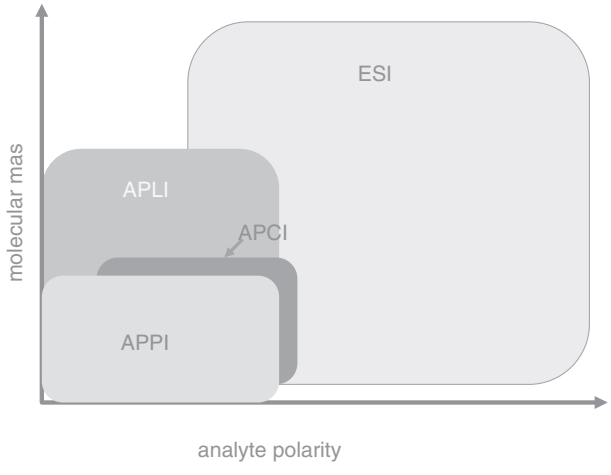


Figure 1.2 Polarity range of analytes for ionization with various API techniques. Note: the extended mass range of APLI against APPI and APCI results from the ionization of nonpolar aromatic analytes in an electrospray.

1.1.2.1 Overview about API Methods

Ionization methods that operate at AP, such as the APCI and the ESI, have greatly expanded the scope of mass spectrometry [16–19]. These API techniques allow an easy coupling of chromatographic separation systems, such as liquid chromatography (LC), to a mass spectrometer.

A fundamental difference exists between APCI and ESI ionization mechanisms. In APCI, ionization of the analyte takes place in the gas phase after evaporation of the solvent. In ESI, the ionization takes place already in the liquid phase. In ESI process, protonated or deprotonated molecular ions are usually formed from highly polar analytes. Fragmentation is rarely observed. However, for the ionization of less polar substances, APCI is preferably used. APCI is based on the reaction of analytes with primary ions, which are generated by corona discharge. But the ionization of nonpolar analytes is very low with both techniques.

For these classes of substances, other methods have been developed, such as the coupling of ESI with an electrochemical cell [20–31], the "coordination ion-spray" [31–46], or the "dissociative electron-capture ionization" [37–41]. The APPI or the dopant-assisted (DA) APPI presented by Syage *et al.* [42, 43] and Robb *et al.* [44, 45], respectively, are relatively new methods for photoionization (PI) of nonpolar substances by means of vacuum ultraviolet (VUV) radiation. Both techniques are based on photoionization, which is also used in ion mobility mass spectrometry [46–49] and in the photoionization detector (PID) [50–52].

1.1.2.2 ESI

In the past, one of the main problems of mass spectrometric analysis of proteins or other macromolecules was that their mass was outside the mass range of most mass spectrometers. For the analysis of larger molecules, such as proteins, a hydrolysis and the analysis of the resulting peptide mixture had to be carried out. With ESI, it is now possible to ionize large biomolecules without prior hydrolysis and analyze them by using MS.

Based on previous works from Zeleny [53], and Wilson and Taylor [54, 55], Dole and co-workers produced high molecular weight polystyrene ions in the gas phase from a benzene/acetone mixture of the polymer by electrospray [56]. This ionization method was finally established through the work of Yamashita and Fenn [57] and rewarded in 2002 with the Nobel Prize for Chemistry.

The whole process of ion formation in ESI can be subdivided into three sections:

- formation of charged droplets
- reduction of the droplet
- formation of gaseous ions.

To generate positive ions, a voltage of 2–3 kV between the narrow capillary tip (10^{-4} m outer diameter) and the MS input (counter electrode) is applied. In the exiting eluate from the capillary, a charge separation occurs. Cations are enriched at the surface of the liquid and moved to the counter electrode. Anions migrate to the positively charged capillary, where they are discharged or oxidized. The accumulation of positive charge on the liquid surface is the cause of the formation of

a liquid cone, as the cations are drawn to the negative pole, the cathode. This so-called Taylor cone resulted from the electric field and the surface tension of the solution. At certain distance from the capillary, there is a growing destabilization and a stable spray of drops with an excess of positive charges will be emitted.

The size of the droplets formed depends on the

- flow rate of the mobile phase and the auxiliary gas
- surface tension
- viscosity
- applied voltage
- concentration of the electrolyte.

These drops loose solvent molecules by evaporation, and at the Raleigh limit (electrostatic repulsion of the surface charges > surface tension) much smaller droplets (so-called microdroplets) are emitted. This occurs due to elastic surface vibrations of the drops, which lead to the formation of Taylor cone-like structures.

At the end of such protuberances, small droplets are formed, which have significantly smaller mass/charge ratio than the "mother drop" (Figure 1.3). Because of the unequal decomposition the ratio of surface charge to the number of paired ions in the droplet increases dramatically per cycle of droplet formation and evaporation up to the Raleigh limit in comparison with the "mother drops." Thus, only highly charged microdroplets are responsible for the successful formation of ions. For the ESI process, the formation of multiply charged ions for large analyte molecules is characteristic. Therefore, a series of ion signals for, for example, peptides and proteins can be observed, which differ from each other by one charge (usually an addition of a proton in positive mode or subtraction of a proton in negative mode).

For the formation of the gaseous analyte, two mechanisms are discussed. The charged residue mechanism (CRM) proposed by Cole [58], Kebarle and Peschke [59], and the ion evaporation mechanism (IEM) postulated by Thomason and Iribarne [60]. In CRM, the droplets are reduced as long as only one analyte in the microdroplets is present, then one or more charges are added to the analyte. In IEM, the droplets are reduced to a so-called critical radius ($r < 10\,\text{nm}$)

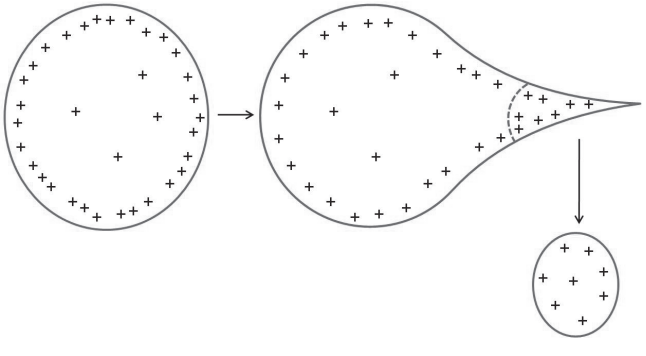


Figure 1.3 Reduction of the droplet size.

and then charged analyte ions are emitted from these drops [61]. It is essential for the process that enough charge carriers are provided in the eluate. This can be realized by the addition of, for example, ammonium formate to the eluent or eluate. Without this addition, ESI is also possible with an eluate of acetonitrile/water (but not with MeOH/water), but a more stable and more reproducible electrospray with a higher ion yield is only formed by adding charge carriers before or after high-performance liquid chromatography (HPLC) separation.

1.1.2.3 **APCI**

This ionization method was developed by Horning in 1974 [62]. The eluate is introduced through an evaporator (400–600 °C) into the ion source. Despite the high temperature of the evaporator, a decomposition of the sample is only rarely observed, because the energy is used for the evaporation of the solvent, and the sample is normally not heated above 80–100 °C [63]. In the exit area of the gas flow (eluate and analyte), a metal needle (Corona) is mounted to which a high voltage (HV) is applied. When the solvent molecules reach the field of high voltage, a reaction plasma is formed on the principle of chemical ionization. If the energy difference between the analyte and reactant ions is large enough, the analytes are ionized, for example, by proton transfer or adduct formation in the gas phase.

For the emission of electrons in APCI, a corona discharge is used instead of the filament in GC-MS (CI) because of the rapid fusion of the filament at AP. In APCI, with nitrogen as sheath and nebulizer gas and atmospheric water vapor (also in 5.0 nitrogen sufficient quantity of water is available), $N_2^{+}\cdot$and $N_4^{+}\cdot$ ions are primarily formed by electron ionization. These ions collide with the vaporized solvent molecules and form secondary reactant gas ions, such as H_3O^+ and $(H_2O)_nH^+$ (Figure 1.4).

The most common secondary cluster ion is $(H_2O)_2H^+$, together with significant amounts of $(H_2O)_3H^+$ and H_3O^+. These charged water clusters collide with the analyte molecules, resulting in the formation of analyte ions:

$$H_3O^+ + M \rightarrow [M + H]^+ + H_2O$$

The high collision frequency results in a high ionization efficiency of the analytes and adduct ions with little fragmentation. In the negative mode, the electrons that are emitted during the corona discharge form – together with large amounts of N_2 and the presence of water molecules – OH– ions. Due to the fact that the gas phase acidity of H_2O is very low, OH^- ions in the gas phase form by proton transfer reaction with the analyte H_2O and $[MH]^-$ (M = analyte) [63]. The problem with

$$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e^-$$
$$N_2^{+\cdot} + 2N_2 \rightarrow N_4^{+\cdot} + N_2$$
$$N_4^{+\cdot} + H_2O \rightarrow H_2O^{+\cdot} + 2N_2$$
$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^{+\cdot} + OH^{\cdot}$$

$$H_3O^+ + n\,H_2O \rightarrow H_3O^+ \cdot (H_2O)_n$$

Figure 1.4 Reaction mechanism in APCI.

APCI is the simultaneous formation of different adduct ions. Depending on eluent composition and matrix components, it is possible that Na^+ and NH_4^+ adducts can occur besides protonated analyte molecules, making the data evaluation more difficult.

1.1.2.4 APPI

APPI is suitable for the ionization of nonpolar analytes, in which the photoionization of molecule M leads to the formation of a radical cation $M^{\bullet+}$. If the ionization potentials (IPs) of all other matrix elements are greater than the photon energy, then the ionization process is specific for the analyte. However, in the APPI, different processes can very strongly influence the detection of $M^{\bullet+}$:

1) In the presence of solvent molecules and/or other existing components in large excess, ion-molecule reactions can proceed.
2) VUV photons are efficiently absorbed from the gas phase matrix.

Thus, for example, in the presence of acetonitrile (often used mobile phase in HPLC), mainly $[M + H^+]$ is formed even though the IP of acetonitrile is more than 2.2 eV higher as the photon energy [64]. In general, in the case of polar compounds, which are dissolved in CH_3CN/H_2O, the formation of $[M + H]^+$ is usually observed, while nonpolar compounds such as naphthalene usually form $M^{\bullet+}$ [65]. A detailed mechanism for the formation of $[M + H]^+$ was proposed by Klee *et al.* [66]. In APPI, the ion yield is reduced due to the limited VUV photon flux, and the interactions with solvent molecules. Therefore, the DA-APPI was introduced as a new ionization method from Bruins and employees [65].

The total number of ions, which are formed by the VUV radiation, is significantly increased by the addition of a directly ionizing component (dopant). If the dopant is selected such that the resulting dopant ions have a relatively high recombination energy or low proton affinity, then the dopant ion can ionize the analytes by charge exchange or proton transfer. In addition to acetone and toluene also anisole was found to be a very effective dopant in APPI [67]. By adding dopants the sensitivity can be increased, but the possible adduct formations often lead to significantly more complicated APPI mass spectra [44, 65, 67]. Recent studies suggest that the direct proton transfer from the initially formed dopant ions plays only a very minor role, and the ionization process is dominated by a very complex, thermodynamically controlled cluster chemistry.

1.1.2.5 APLI

APLI was developed in 2005 [68]. It is a soft ionization method with easy-to-interpret spectra for nonpolar aromatic substances and only minor tendency for fragmentation of the analytes. APLI is based on the resonance-enhanced multiphoton ionization (REMPI), however, at AP. The REMPI method allows the sensitive and selective ionization of numerous compounds. Here, for example, the following approach is used:

$$M + mh\nu \rightarrow M* \qquad \text{(a)}$$

$$M* + nh\nu \rightarrow M^{\cdot +} + e^{-} \quad \text{(b)}$$

Reactions a and b represent a classical $(m+n)$ REMPI process, where $n=m=1$ is often very beneficial for the ionization of PAH. Because the absorption bands of PAHs are relatively broad at room temperature, and PAHs have high molecular absorption coefficient in the near ultraviolet and a relatively long lifetime of the S1 and S2 states a fixed-frequency laser, for example, the 248 nm line of a KrF excimer laser, can be used. Under these conditions, an almost selective ionization of aromatic hydrocarbons can be achieved.

A great advantage of APLI in comparison to APPI is that neither oxygen nor nitrogen and the solvents typically used in the HPLC (e.g., water, methanol, acetonitrile) have appreciable absorption cross-sections in the used wavelength range. An attenuation of the photon density within the ion source, that is, a significant coupling of electronic energy into the matrix, as observed in the APPI, does not take place in APLI. The APLI is very sensitive in the determination of PAHs and, therefore, represents a valuable alternative to APCI and APPI, but APLI is not only restricted to the analysis of such simple aromatic compounds. Also, more complex oligomeric or polymeric structures and organometallic compounds can be analyzed [69]. It is also possible to analyze nonaromatic compounds after derivatization of their functional group with so-called ionization markers, in analogy to fluorescence derivatization [70]. With this technique, you can benefit from the selectivity of the ionization (only aromatic systems) and the outstanding sensitivity of the method. In addition, a parallel ionization of sample components with ESI or APCI together with APLI was realized [71, 72] to analyze polar (ESI) or nonaromatic medium polar (APCI) compounds together with aromatic (APLI) compounds.

1.1.2.6 Determination of Ion Suppression

In many mass spectrometric analysis of complex samples, the ion suppression leads to a more difficult quantitative determination and often time-consuming sample preparation is required. It should, therefore, be studied more in advance whether there is a signal-reducing influence of the matrix.

For the investigation of ion suppression, the sample solution (without analyte) is injected in the HPLC and a solution with the analyte (stable-isotopic labeled analyte, if no sample solution without analyte is available) is mixed behind the separation column via a T-piece to the eluate, and the mass trace of the analyte (or stable-isotopic labeled analyte) is analyzed during the total analysis time. After the column, the separated matrix ingredients are mixed with the analyte in the T-piece and are transported into the ion source. The change in intensity of the analyte mass trace before and after the injection of the matrix provides information about a possibly occurring ion suppression.

Figure 1.5 shows the determination of ion suppression of a PAH analysis in urine with APCI-quadrupole-time of flight (QTOF). During the analysis time between 80 and 400 s, the mass trace is considerably diminished and reaches the normal

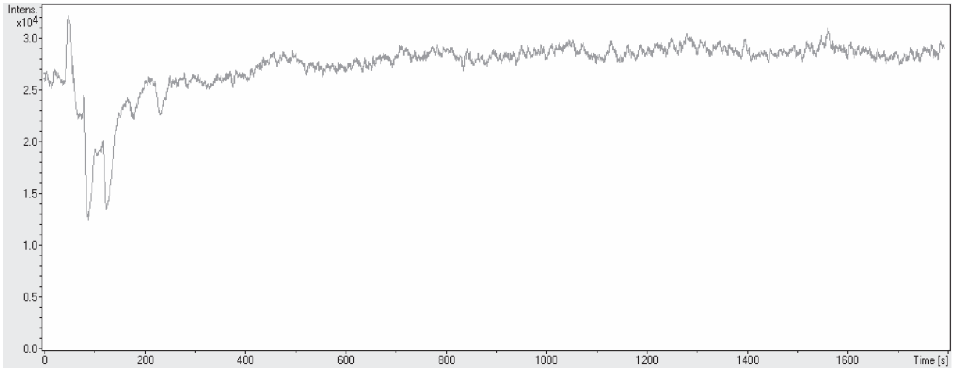


Figure 1.5 Ion suppression in APCI-MS of PAH in urine.

level after about 450 s. This means that between 80 and 400 s disturbing matrix components of urine left the column, which leads to ion suppression.

1.1.2.7 Best Ionization for Each Question

On the basis of Figure 1.2, the method can roughly estimate which allows the most effective ionization for the analyte of interest. Depending on the polarity of the analyte, the ionization should be done with ESI (polar analytes), APCI (moderately polar analytes), APPI (nonpolar analytes), or with APLI (aromatics). However, the matrix plays an important role in making this decision. For complex samples, an ion suppression with ESI is more likely and more pronounced than for the other ionization methods discussed here. The ion beam line also plays an important role in the inlet region of the mass spectrometer. ESI ion sources with a Z-spray inlet often show less ion suppression than normal ESI ion sources. Also, the eluate flow must be adapted to the ion source. For example, slightly higher fluxes than with ESI sources can often be used in APCI sources. Although MS manufacturers promise other flow rates, so it is with regard to spray stability, reproducibility and ion suppression useful to operate ESI sources with fluxes below 300 µl/min and APCI, APPI, and APLI sources with fluxes below 500 µl/min. Of course, due to the application even larger flows can be used, but often problems such as ion suppression or spray instability are observed.

1.1.3 Mass Analyzer

The most frequent mass spectrometers, which are routinely coupled to the LC, are as follows:

- Quadrupole
- TripleQuad
- IonTrap
- oaTOF
- Orbitrap.

With regard to sensitivity and ratio of price and performance (including maintenance), a quadrupole MS is a very good purchase. With single ion mode (SIM), a very good sensitivity can be achieved and a fast quadrupole (from about 25 to 50 Hz) allows the coupling with a fast ultra-high-performance liquid chromatography (UHPLC) separation.

Based on quadrupole MS, a further development represents the triple quadrupole mass spectrometer, which plays an increasingly important role, especially in the target analysis in complex samples. The sample preparation is minimized, a preliminary separation is often omitted and the potentials of the first and third quadrupole are adjusted so that only a certain mass is allowed to pass these quadrupoles. In the first quadrupole, the ion of the target analyte and in the third quadrupole a characteristic ion fragment, which is induced by collisions with argon in the second quadrupole, is passed through. Due to the analysis of the fragment ion, the chemical noise (matrix) is greatly reduced and triple quadrupole mass spectrometer are one of the most sensitive and selective mass spectrometers. Detection limits in zeptomoles area (amount of substance on the separation column) have been realized for some analytes.

Similar to a quadrupole, an ion trap is constructed. However, the ions are collected in the trap, and then, either a mass scan or single to multiple fragmentation of the target analyte can be performed. Modern ion-trap MS systems are characterized by a very good linearity and sensitivity and a fast data acquisition (e.g., 20 Hz) and thus can even be coupled with UHPLC. They are particularly suitable for structure determination of biomolecules (carbohydrates, peptides, etc.).

For more than 20 years the use of time-of-flight (TOF) mass spectrometer is increasing, which is related to the orthogonal ion beam guiding in the device. The orthogonal ion beam has made it possible to couple even continuous ion sources, such as ESI and APCI, without loss of resolution to a TOF-MS. Recently, the resolution was steadily improved through the introduction of repeller electrodes, ion funnels, more powerful electronics, and so on, so that now several manufacturers offer TOF-MS systems with resolutions between 40 and 50 000 while realizing data acquisition rates of 20 Hz or more. Thus, these devices are ideally suitable for the coupling of fast separation techniques such as UHPLC and can also provide assistance in the identification of unknown sample components due to the high resolution and mass accuracy (<1 ppm).

One of the latest mass analyzer is the linear-trap quadrupole (LTQ) Orbitrap mass spectrometer. In this, the commercial LTQ is coupled with an ion trap, developed by Makarov [73, 74]. Due to the resolving power (between 70 000 and 800 000) and the high mass accuracy (2–5 ppm), Orbitrap mass analyzers, for example, cab be used for the identification of peptides in protein analysis or for metabolomic studies. In addition, the selectivity of MS/MS experiments can be greatly improved. However, the coupling is not useful with UHPLC for rapid chromatographic pre-separation, as the data acquisition rate is too low for a reproducible integration of the narrow signals produced with UHPLC.

In addition to some other mass spectrometers, FTICRMS devices are also used. The latter, in addition to very high acquisition and operating costs (e.g., helium), has the disadvantage of low data acquisition rate (same problem as with the Orbitrap), so the coupling with a fast analysis, such as UHPLC, cannot be realized. However, they are unbeaten in resolution and an extremely useful tool in metabolomic research.

1.1.4 Future Developments

The trend in mass spectrometry is currently clearly toward higher resolution and faster data acquisition.

Probably, in future, resolution of about 100 000 and data rates of 20 – 40 Hz can be achieved with TOF-MS. With Orbitrap-MS, it is assumed that resolutions of more than 800 000 will be possible by more precise production of the cell and electronic devices. This would make it possible to reduce the scanning speed and then to realize the coupling with UHPLC also with good mass resolution.

By connecting an ion mobility spectrometry (IMS) in front of a QTOF-MS, another dimension of separation is realized. Unseparated isobaric compounds, which have the same m/z value, can be separated after ionization by the structure-dependent drift time through the IMS. The combination of IMS with QTOF is also a powerful tool for nontarget analysis in complex samples, due to the fact that the chemical noise is drastically reduced by IMS.

Another focus in future developments will be the optimization of ion sources with respect to ion generation and ion transport at different flows, which are used in nano- and micro-HPLC, LCxLC, and SFC to increase the sensitivity.

1.1.5 What Should You Look for When Buying a Mass Spectrometer?

In addition to the available budget in my opinion, the following points play a central role in making buying decisions:

- a target analysis or a comprehensive analysis of the sample are carried out
- needed sensitivity
- software
- sample throughput
- MS analysis with or without pre-separation process.

If only target analyses is planned (e.g., analysis of known impurities in a product or pesticide analysis), a quadrupole or triple quadrupole-MS would be the best choice. With these devices a very sensitive analysis will be guaranteed, and also a quick pre-separation (e.g., UHPLC) is now possible for many devices.

If nontarget analysis should be realized, high-resolution mass spectrometer such as QTOF or Orbitrap would facilitate the analysis considerably. Even if a high sample throughput is still necessary, the QTOF would get precedence over the slow Orbitrap in high-resolution mode. However, regarding the resolution Orbitrap, in comparison with QTOF, is the more powerful system. The sensitivity of a QTOF is about a factor 10 lower than that of a triple quad, but detection limits in the lower parts per billion range are quite possible.

Perhaps, due to a high number of samples, no pre-separation will be done. But then, it should be ensured that suitable so-called ambient desorption ionization techniques such as DESI, DART, ASAP, and DIP-APCI can be coupled to the MS.

Finally, there are large differences in the respective MS software. Here, the user should provide an overview of the strengths and weaknesses of the software.

In addition to the price of the system, operating costs should also be considered. Besides a high nitrogen consumption, the mass spectrometer should be serviced annually. Just the maintenance leads depending on the effort and manufacturer to an annual cost of €5 – 20 000.

1.2 Technical Aspects and Pitfalls of LC/MS Hyphenation

Markus M. Martin

For almost two decades, the coupling of liquid chromatography (LC) and mass spectrometry (MS) has left the stage of breadboard lab designs and is commercialized with manifold off-the-shelf products. Frankly, the first systems on the market required a strong expertise and highly skilled users and thus were exclusively applied in highly specialized research laboratories; however, due to intensive research and development work, the robustness and ease of use of LC/MS systems have improved so much over the years that LC/MS techniques are established meanwhile even in many routine applications. Considering how different the two worlds of a separation in the liquid phase via LC and in the gas phase via MS are, this is truly a remarkable fact. Both liquid chromatographs and mass spectrometers have meanwhile achieved a high degree of technical perfection that allows even the less experienced users to create reliable results in a fairly short learning time; nevertheless, the list of potential error sources in the LC/MS hyphenation is still long these days. It starts with the selection of an unsuitable Instrumentation and does not yet end with the wrong interpretation of experimental results. Some errors are specific for instruments, methods, or applications – think, for instance, of the countless variants of matrix effects in the field of food analysis; their individual discussion is beyond the scope of this section. Other aspects are more of a general or fundamental nature – this is what is discussed in this chapter.

1.2.1 Instrumental Considerations

1.2.1.1 Does Your Mass Spectrometer Fit Your Purpose?

It is a long-stressed platitude that the right tool makes all the difference: anyone who has ever tried to fix an inch hex bolt with a metric wrench will confirm this from personal experience. Well, what applies to screwdrivers is in fact not different to high-tech analysis equipment in your lab, and it is particularly correct for mass spectrometry. Currently, five different mass analyzer principles are established in the market for LC/MS applications:

- Quadrupole (Q)
- Ion trap (IT)
- Time of flight (TOF)
- Orbitrap
- Ion cyclotron resonance (ICR).

Nearly all commercial LC/MS instrumentations rely on (at least) one of those five mass selectors; more sophisticated devices may either vary slightly in their technical design (e.g., 3D or Paul trap, quadrupole ion trap (*QIT*), vs. linear ion trap (*LIT*)), or come as hybrid instruments combining two or more of these analyzer types (e.g., Triple Quadrupole, *QqQ*, *Qq-TOF*, Ion trap-Orbitrap, *LIT*-Orbitrap, or even Tribrids merging 3 different analyzers into one device). Each of those solutions has its strengths and weaknesses, which make it more appropriate for certain applications than for others. The previous chapter of this textbook gives a comprehensive overview on the technological state of the art; for additional information refer also to [75, 76].

But whatever field of application you are looking for – nearly every analytical challenge requiring mass spectrometric detection can be reduced to either one of the two aspects:

- selective detection of previously known analytes with highest sensitivity for quantitation, *or*
- identification and structure elucidation of unknown compounds.

Combining these tasks with the technical potential of UHPLC, which enables ultra-high separation performance and/or high speed of analysis, will then result in a very attractive technology for the fast *and* comprehensive screening of complex samples with low sample preparation efforts (*dilute-and-shoot*) and high throughput. However, the capabilities of a mass spectrometer need to keep pace with the increasing requirements dictated by higher sample complexity and shorter analysis times. Of course, you can apply a given mass analyzer type also to analytical questions where it would not be your spontaneous first choice. For instance, nothing speaks against the use of a single-quad mass spectrometer to quantify targeted analytes in a fairly simple sample of low complexity. Most single-quads achieve very low limits of quantitation (LoQ) when run in the *single-ion monitoring (SIM)* mode; and as long as you can be sure that only one

analyte species exists with your given target mass, this – rather low-tech – mass spec type can deliver reliable results. Or, to stress another extreme: in case of the measuring time not being a limiting factor, on principle you could also (mis)use an Fourier transform ion cyclotron resonance (FTICR) mass spectrometer for a super-sensitive routine screening analysis, although this would be a decadent waste of money given the immense investments you would need to make. However, you will get the highest confidence in your result if you apply the most suited mass spectrometer to a given analytical problem. Let us briefly discuss now the pros and cons of the various mass spectrometer types for our two core analytical tasks mentioned earlier, either the quantitation of known analytes as specific and sensitive as possible (*Targeted Screening*), or the fishing in the troubled water of samples where you do not have a clue about what compounds to expect (*Screening for Unknowns*).

For **Targeted Screening**, with a clear focus on quantitation of previously known target analytes, all those MS types are preferred that combine two mass analyzers with a collision cell in-between, allowing for *collision-induced dissociation (CID)* by *tandem-MS in space*. From all potential MS/MS operation modes offered by these instrument types, Targeted Screening is most frequently run in the *Selected Reaction Monitoring* (*SRM,* also called *Multiple Reaction Monitoring, MRM)* mode. This operation mode requires that you have a good understanding of how your target analyte dissociates into characteristic and ideally specific fragments after exciting it to vibrations by collision with an inert gas in a collision cell. For the collision gas, the heavier argon is typically preferred over the light nitrogen for a higher kinetic impact. You will operate the two mass analyzers as ion filters then; the first one in front of the reagent cell eliminates all unwanted ions so that only the ions with an m/z value of your target analyte, the *precursor ions*, enter the collision cell. The second mass filter behind the cell then is set to the m/z value(s) of the expected *fragment ions*. This SRM operation mode features two main advantages: the combination of precursor ion with as many characteristic fragment ions as possible substantially increases the detection specificity, and it ensures tremendously low limits of detection (LoD) and quantitation. Running the MS in SRM mode not only filters out all unwanted interfering ions, thus virtually eliminating baseline noise; it also reserves the full MS duty cycle exclusively for the detection of the target analyte ions, allowing you to detect a much higher amount of your target ions than in a full scan mode. Up to now, triple quadrupole mass spectrometers (QqQ) are the uncrowned leaders in the Targeted Screening domain, being superior to Qq-TOF or other instrument types with respect to sensitivity, ease of use, result robustness, and profitability. Particularly, ion-trap mass spectrometers, which basically offer the inherent advantage of *tandem MS and* MS^n *in time* for even more specific fragmentation experiments, are not ideal for quantitation purposes due to their limited linear detection range (refer also to the *space charge* phenomenon in Section 1.2.3.5). In addition, ion traps will completely fail for all MS/MS operation modes that require a scan process as the first step in a row of MS experiments due to their operation principle. If you need to perform a "true" *precursor ion scan* or a

constant neutral loss scan for your analysis (and not merely a software data set reconstructing these scan modes out of a set of sequential MS^n experiments), then the use of a *tandem MS in space* machine such as a triple quad device is imperative. It should be noted that depending on the molecular mass of your target analytes, the preferred MS/MS instrument type may slightly vary. For small molecules of typically less than 1000 – 1200 Da, a triple quad machine clearly rules out other MS types due to its benefits in robustness, sensitivity, and investment costs. However, the comparably low upper m/z limit of QqQ's is of slight disadvantage; hence, Qq-TOF and Orbitrap instruments are more in favor for large and macromolecules.

The other focus for LC/MS applications is the **Screening for Unknowns**, where you primarily need to learn about unknown sample constituents as much as you can with a very low experimental effort – ideally within one single LC/MS injection. The most relevant information you would need to gather comprises

- *the elemental composition* – which can be derived from high resolution/accurate mass (HR/AM) measurements
- *molecular substructures* – to be determined by MS/MS or MS^n experiments
- the *signal intensity ratio of the isotopes*, the so-called isotope pattern, which backs up the elemental composition calculation based on HR/AM results.

As already discussed in Section 1.1.1, only TOF, Orbitrap, and FTICR mass analyzers allow for reliable HR/AM measurements with a sufficient mass accuracy of less than 5 ppm and resolving power. Coupling these analyzers with a quadrupole and a collision cell upfront enables you to additionally measure CID fragment spectra revealing details on molecular substructures, functional groups, and so on, thus supporting structure elucidation. Data acquisition speed and resolving power R behave strictly opposite within these three MS types: as of today, TOF devices are by far the fastest mass spectrometers on the market (max. scan rate of up to 200 Hz), followed by Orbitraps (up to 18 Hz) and FTICR (1 Hz or less); In contrast, FTICRs lead in terms of resolving power (R up to 10 000 000), followed by Orbitraps (R up to 500 000) and TOFs (R up to 80 000).

- *TOF devices* offer exciting scan speeds, high mass accuracy, and resolving power at a good price per performance; however, they tend to be very prone even to minor variations of the environmental conditions. As with all materials, also the flight tube of a TOF-MS expands with higher temperature. An elongation (or shrinking) of the flight tube even only on the micrometer scale will substantially affect the accuracy of the mass determination (to be precise: the mass/charge determination) and the resolving power. For a stable and rugged experimental result, you will need a powerful and precise air conditioning in your MS lab (be also aware of sun glare shining on the mass spectrometer through the windows!) as well as a regular mass calibration, for example, on a 1 h frequency or even more often, to compensate for any drifts. As a drifting mass axis calibration can easily occur already on the timescale of one LC separation, the highest confidence in your mass accuracy can only be guaranteed by an internal

mass calibration where known mass calibration compounds are permanently co-infused into the MS during the LC run. Some TOF devices offer a continuous calibrant infusion as a *lock spray* into the ion source using a revolving aperture that alternatingly passes either the LC effluent or the calibrant solution into the mass analyzer. As an alternative, the calibrant solution can also be added to the LC effluent in front of the ion source by a simple tee piece setup. For a correct data analysis, every measured m/z value taken from the LC/MS data set is then referenced against the m/z values of the known calibrants. This may sound a bit clumsy, but it is the only way for TOF devices to fully reach their maximum specified mass accuracy.

- *Orbitrap devices*, in contrast, are much more rugged against changes in the ambient conditions due to their inherently different design and operation principle. For routine applications, a mass calibration once a week is typically sufficient (depending on the application and lab conditions). Next to the higher analysis ruggedness, they are significantly superior to TOF devices in terms of mass resolution and at least par with respect to mass accuracy – in fact they are the only mass analyzers that come even close to the accuracy of FTICR but with much less challenging claims for technical infrastructure, as they are true benchtop instruments today.
- *FTICR instruments* are very, very sensitive, being capable to detect even down to 10 individual molecules within their detection cell; and they are unbeaten yet in terms of mass resolving power and mass accuracy. However, the very low data rate, the limited linear detection range, their bulky size, and last but not least the massive total costs of operation (think not only of the device alone but also of the demanding infrastructure for the superconductive magnet) will make this mass spectrometer type a highly dedicated expert system also in the foreseeable future, asking for a high level of user expertise and by that not having a real chance to establish themselves in routine applications.
- *Ion traps* (being the only MS type together with FTICR) featuring tandem MS in time and by that MS^n experiments with $n \geq 2$ are the most versatile instruments for substructure elucidation by gas phase fragmentation reactions. Due to their limited mass accuracy of typically greater than 10 ppm and only moderate resolving power, they are not really suitable for HR/AM analyses. Their preferred field of application is, therefore, the elucidation of analyte structures for compound classes with only a limited variability of building blocks, such as the analysis of peptides, proteins, and nucleic acids.

A rather special position in the MS world is held by the fairly simple single quadrupole mass spectrometers. With their low mass accuracy (>100 ppm) and quite poor resolving power (R about 1000 for $m/z = 1000$), they are neither good for structure elucidation/screening for unknowns nor for a specific targeted screening. Their strengths are robustness and a low price, and their mass results can at least support peak assignment during method development and serve as a negative confirmation on the absence of a compound of interest within the limit

Table 1.1 Suitability and purpose of various mass spectrometer types.

	Structure elucidation				
	Elemental composition	Determination of substructures	Screening for unknowns	Simple quantitation	Targeted screening
Q	−	−	−	+	o
QqQ	−	o	o	+	+
QIT	−	+	o	−	o
LIT	−	+	o	o	o
QTRAP	−	+	o	+	+
TOF	+	−	−	o	o
Qq-TOF	+	+	+	o	+
Orbitrap	+	o	o	+	o
Q-Orbitrap	+	+	+	+	+
LIT-Orbitrap	+	+	+	o	+
FTICR	+	+	−	o	−

+ = well-suited; o = moderately suitable; − = inappropriate.

of detection. Therefore, they are frequently used as screening detectors with samples of low complexity, for instance, in the open-access process control analysis of combinatorial reactions. Due to their limited mass spectrometric performance, many users do not even perceive single quads as true mass spectrometers but much more as mass-selective detector (MSD), a concept that is meanwhile widely adopted by the marketing activities of various single quad manufacturers.

Table 1.1 gives a rough overview on the suitability of most common mass spectrometer types of today in combination with UHPLC for various application scenarios. In addition to the earlier discussed Targeted Screening and Screening of Unknowns, more generalized aspects of structure elucidation and quantitative amount determination are listed as well. It should be mentioned that this table has of course to live with a certain generalization. All major instrument manufacturers may offer individual, highly specialized flavors of the one or the other type of mass analyzer, which exceeds the general limitations predicted by this list, but from a general perspective, this categorization applies very well to the different mass spectrometer capabilities and applicability.

1.2.1.2 (U)HPLC and Mass Spectrometry

UHPLC has meanwhile been widely accepted and established in the last years, for both LC standalone and LC/MS workflows. UHPLC is highly attractive to mass spectrometry detection due to either the gathering of the same analytical information as a conventional HPLC separation in much shorter time or, thanks to a significantly improved chromatographic resolution, collecting much more information on your sample in a given time. A shot-run method, specially designed for very fast analyses, enables high-throughput screening (HTS) and improves both workload and payback period of a mass spectrometer.

A high-resolution separation, however, that avoids co-elution of analytes reduces competing ionization and ion suppression in the MS ion source, resulting in higher sensitivity and better spectra quality (cf. also Section 1.2.3.3). But it is exactly this UHPLC potential of high speed and efficiency that requires a thorough optimization of your instrumentation to ensure that the high separation performance of your UHPLC column is translated lossless into a perfect LC/MS chromatogram.

Speed in LC/MS Analysis I: Struggling with the Gradient Delay HTS is one focus area for LC/MS applications, for instance, in drug research and development in the pharmaceutical industry. Analysis times of less than 2–5 min for samples of modest complexity enable the fast and reliable processing even of large sample pipelines in an uninterrupted 24/7 routine operation, which makes this approach highly attractive, for example, for combinatorial synthesis monitoring or drug metabolism and pharmacokinetics studies (DMPK). With such short analysis cycles, the gradient delay volume (GDV) of a UHPLC system becomes a critical factor for the overall sample throughput. The GDV is defined as the sum of all volume contributions from the point of gradient formation to the column head. Hence, the GDV has a major impact on the appearance of a chromatographic gradient separation; it is the reason for any gradient separation to begin with an isocratic hold-up step, which takes as long as a change in the mobile phase composition needs to reach the column head and to interfere with the separation process. LC/MS applications in particular ask for separation columns with small inner diameters (from 2.1 mm I.D. columns for analytical scale separations down to several dozens or hundreds of microns in nano- and cap-LC applications), which come along with downscaled flow rates of less than 1 ml/min, with typical values between 50 and 500 µl/min. A small GDV is of high advantage here: the fastest gradient program is useless if a GDV of 500 µl in combination with a flow rate of 500 µl/min makes the changed eluent composition arrive at the UHPLC column head with a delay of one full minute. And please do not get blinded by smart marketing messages of the instrument vendors, which in most cases only specify the mixer size of the (U)HPLC pump: of course, the mixer volume is part of the GDV, but the total GDV amount will be much more than that; it includes the sample loop and other fluidic parts of the autosampler as well as all connecting tubing or, for instance, the whole pump head fluidics in case you are using a low-pressure gradient (LPG) pump. Therefore, a small mixing volume only pays off if it provides sufficient mixing efficiency together with the entire rest of the (U)HPLC system also matching the fluidic requirements for fast LC.

Pump type and mixing volume: To some degree, all modern (U)HPLC pumps allow you to realize an overall GDV of 250 µl or less – getting much below 100 µl of GDV, however, is still a major challenge. Due to their operation principle, *high-pressure gradient (HPG) pumps* have an inherent advantage with respect to GDV

compared with *LPG pumps*; this makes a HPG pump the preferred one particularly for LC/MS applications. Using an LPG limits your LC method speed-up capabilities for LC and LC/MS applications significantly; this can only be overcome by reducing all potential GDV contributions, for example, by installing a smaller eluent mixer. But, wouldn't the mixing efficiency suffer from such a mixer change? Well, it depends on which perturbation effect would be affected, the *radial mixing* (i.e., along the cross section of your fluidics) or the *longitudinal or axial mixing* (along the flow direction) of your mobile phase. Radial mixing is regularly achieved by complex shifts and changes in the liquid stream, for instance, induced by a mixing helix or by branched channel structures on a chip-design mixer. Radial mixing is most required by HPG pumps due to their operation principle, and fortunate enough this needs only a small mixing volume. Axial mixing in contrast is most effectively achieved by larger mixing volumes. Unfortunately, it is the LPG pump operation principle that asks mostly for axial mixing. As a consequence, reducing the volume of your mixing device will have much less of an impact on the performance of an HPG than of an LPG. In addition, baseline stability, drift, and noise, suffers less from an axial inhomogeneity of the mobile phase in MS detection than in UV detection. All these are good arguments that a small mixing volume combined with an HPG pump is much less of a problem for LC/MS applications than it is for standalone (U)HPLC.

The GDV discussion is a particularly difficult one for pumps still having membrane-based pulsation dampeners. In this case, the GDV also depends on the system pressure, and by that on the separation flow rate [77]. While all major manufacturers of modern UHPLC pumps nowadays have established electronic control mechanisms in their high-end and most middle-class instruments that allow a virtually ripple-free flow delivery without dampeners, some older pumps or simpler entry-level models still have to rely on mechanical dampening. It would not be appropriate in general to use such pump types together with MS detection.

But what to do if you cannot further reduce the GDV of your system but still want to profit from a very short and steep, a *ballistic* gradient separation? Well, a workaround can be a delayed sample injection. It sounds simple: your autosampler does not inject the sample simultaneously with the pump starting the gradient program, but the sample is introduced with a certain time delay, which equals the GDV to be saved at the programmed flow rate. This operation principle is especially applicable if you need to transfer a separation method coming from a system with a lower GDV than yours, as it allows you to reduce the effective isocratic hold-up the sample goes through after injection. But do not be deceived – this is beneficial if you look on one single chromatogram, as it reduces the overall data acquisition time for this run: data recording still starts with the time of injection, not with the start of the pump program. However, it is the total run time for this separation, your cycle time, which still remains the same. Thus, delayed injection is a nice workaround for method transfer, but it will not help you to increase your sample throughput.

Sample Injection: Another important contributor to the GDV is the autosampler, which offers a lot of optimization potential. Users can typically choose between different sample loop sizes (read: GDV contributions); the default sample loop and system tubing are typically selected in a way that they universally cover nearly every injection volume from low µl to up to 100 µl volumes and more. This requires sample loops of significantly more than 100 µl internal volume. Most UHPLC-MS separations, however, run on 2.1 mm I.D. columns (or less, see also Section 1.2.2.1) and work with much less than 10 µl injection volume to avoid volume and/or mass overloading of the stationary phase. Cutting your sample loop size from nominal 100 µl to less than 30 µl will also reduce the GDV contribution of the autosampler accordingly. Autosamplers with the injection needle being part of the sample loop (*split-loop* principle, also *Flow-through-needle* principle) can additionally benefit from a small-sized needle seat capillary. If your system comes with a motorized high-pressure syringe as part of the sample loop (a *metering device*, as realized, e.g., by Agilent Technologies and Thermo Scientific), this will also contribute to the system GDV. The Vanquish UHPLC systems from Thermo Scientific offer users to modify the GDV setting by a variable metering device piston positioning, which allows a flexible adaptation of the autosampler GDV contribution to your LC separation – a feature that is particularly beneficial for method transfer. And last but not least, many instrument control software offer a bypass mode for autosamplers, which optionally turns the injection valve back to the "load" position after injection. This eliminates the sample loop contribution to the GDV for the rest of the run – a quite significant amount for all split-loop autosamplers. A drawback of this feature is that it cuts a certain volume segment out of a running gradient program, which may have adverse effects on the separation.

System Tubing: A factor that is frequently rather overrated than underrated (in contrast to the topics previously discussed) is the GDV contribution of the system tubing between the pump and the LC column. Particularly, with a bottom-up installation of a modular (U)HPLC system, the connection capillaries can potentially even be slightly longer than in a conventional top-down setup (if, e.g., the degasser is not integrated in the pump module, as exemplified in Figure 1.7b). But no worries – even if a connection line of 0.18 mm I.D. has a length of 19.7″/500 mm, this tube will have "only" 15 µl in volume, which is typically much less than 10% of the total system GDV. You might think that these 15 µl, however, could potentially harm much more as a contribution to band broadening by extra-column volumes (ECVs). Well, this depends on where this tubing is placed. Whenever a larger bore capillary is installed in front of the autosampler, the sample does not encounter it anyway, and peak broadening is no issue at all. And even with wider capillaries positioned between sample loop and column head – the overwhelming majority of LC/MS separations are run in gradient mode. Due to the sample refocusing effect of the gradient program that enriches the analytes on the column head by a huge initial retention at the very low starting solvent strength, the impact of band broadening volumes in

front of the LC column is massively reduced. Hence, any capillary tubing affects noticeably neither the gradient delay nor the band broadening. This statement, however, is only valid for analytical scale LC separations – things look different for capillary and nano LC applications.

Case 1 Take-Home Messages

- Minimize the GDV of your LC system – it will help to remarkably reduce analysis time. HPG pumps are of inherent advantage here.
- GDV means more than the pump mixer. Assess all volumes from the gradient formation point to the column head for minimum GDV, but without sacrificing mixing performance.
- Contributions of connection tubing to the GDV can typically be neglected in case of analytical scale LC separations.

Speed in LC/MS Analysis II: The Total Cycle Time or How Fast Can I Be? As already discussed earlier for the *delayed injection*, it is not the speed of your LC separation alone, typically in gradient mode, which matters for the total cycle time. Various other actions add up to it here, including every step of liquid handling such as sample aspiration, needle wash cycles, or column re-equilibration at the end of your separation.

The first bottleneck for speeding up the total cycle time is already the preparation of the sample injection, as this is fairly time-consuming and depends also on various instrumental properties. Fast state-of-the-art autosamplers can realize injection cycle times of less than 10 – 30 s. This impressive speed, however, can be achieved only with very high sample draw speed and without any external needle wash steps. So, there is a price to be paid: too fast a sample aspiration negatively affects the injection precision, especially with viscous samples or low-boiling sample solvents, while not cleaning the exterior of the injector needle will lead to enhanced carryover effects. Many UHPLC control software offer a "prepare next injection" feature, which already initiates drawing a new sample into the bypassed sample loop while the previous LC separation run is about to be finished, for example, during the column re-equilibration step at the gradient program end. This partial parallelization of injection preparation and chromatographic separation indeed leads to a shortened total cycle time; in real life, however, it is barely possible to achieve a precise and an ultra-low carryover injection in less than 30 s. Besides, this interlacing of injection and analysis steps always requires the sample loop being switched off the fluidic path by turning the injection valve from the *Inject* back to the *Load* position at a certain time in your LC separation. With modern split-loop samplers where the sample loop is a permanent part of the fluidic path, thus ensuring low sample carryover due to a continuous loop rinse by the mobile phase, it is essential that this switch-back takes place only when the sample loop is filled with your initial mobile phase composition. Otherwise, this

will cut a certain volume segment out of your gradient profile, thus actively interfering with the chromatographic process running in your LC column. Hence, an ideal point in time to trigger all kinds of bypassing actions would be during the column re-equilibration step at the gradient end.

The time needed to fill the sample loop can be optimized both by the piston speed of the injection device (typically a glass syringe or a high-pressure piston) and by the injection volume. Thanks to the small injection volumes in UHPLC of less than 1 – 5 µl, a moderate piston draw speed of 250 nl/s still ensures a rapid though reproducible sample dosage even for viscous samples or highly volatile sample solvents. But liquid handling is more than only drawing and injecting dissolved samples. Cleaning internal parts of the sampler fluidics that are in touch with the sample but not continuously flushed by the mobile phase can also become a time-critical step. Some instrument hardware designs use an injection syringe for these internal rinsing steps, and so this cleaning takes the longer the smaller this syringe volume is. Washing a sampler tubing of, for instance, 40 µl with only the fourfold volume of 160 µl wash liquid can take a considerable amount of time, and a 100 µl syringe finishes this cleaning obviously four times faster than a 25 µl syringe. With very unfavorable settings, so in a system with large tubing volume, small cleaning piston volume, and very fast LC separations of less than 2 min of run time, cleaning the autosampler fluidics can even take longer than the entire analytical separation.

But it is not only before or at the beginning of your separation where you have the potential for cycle time optimization. There is also one time-burner at the end of your chromatography, and it can be a substantial one: it is the column re-equilibration. When running a gradient separation, it is imperative to recondition the stationary phase back to the initial mobile phase composition of the gradient once the solvent strength gradient has reached its final level. This is the only way to ensure the mobile and stationary phase being in an equilibrium state, which is a prerequisite for stable retention times. As a rule of thumb, it is recommended to flush the separation column with at least the fivefold column void volume V_m of mobile phase for a stable equilibrium state. With challenging analysis conditions, the required equilibration volume can easily go up to 8 – 10× of the void volume; This is frequently the case either at low initial organic solvent amounts of less than 5% or with analytes strongly affected already by minor deviations from the equilibration state – typically observed for analytes with retention factors of $k < 1$ or for pH-sensitive separations. A short example shall illustrate the time impact here. We will calculate the column void volume V_M from the geometrical column volume V_C using Eq. 1.1:

$$V_M = \varepsilon_t \cdot V_C \tag{1.1}$$

with ε_t = total porosity, r = column radius, L = column length, and $V_C = \pi r^2 \cdot L$

Table 1.2 Recommended re-equilibration volume for high-throughput and high-resolution columns under typical MS-compatible conditions.

Column dimension I.D. × *L* (mm)	V_M (µl)	Required re-equilibration volume (rounded; µl)	Flushing time for 5–8 V_M at 0.5 ml/min
2.1 × 50	113	570–900	1.1–1.8 min
2.1 × 250	563	2800–4500	5.6–9.0 min

Table 1.2 lists two different use cases for comparison, a fairly short UHPLC column for HTS and a long column for a high-resolution analysis, both columns operated at 500 µl/min, which is a good average for a sub-3 µm packing material and still being MS-compatible. You will immediately see that even the HTS column of 2.1 × 50 mm and a typical total porosity of $\varepsilon_t = 0.65$ needs a re-equilibration time of 1.1–1.8 min. The five times longer high-resolution column consequently will require the fivefold of reconditioning time, ending up at between 5.5 and 9 min. With any regular UHPLC system, this amount of time adds to each and every single injection, no matter how fast the gradient separation itself will be. Hence, a total cycle time of less than 2 min is barely achievable. The only way out of this dilemma would be a second separation column of identical properties to the original one, which could be equilibrated in parallel to a running analysis using a second pump and a suitable switching valve. Once the analysis on one column has finished, the next injection is then done alternatingly on the other column, with the previous column being equilibrated simultaneously (Figure 1.6).

Finally, concluding these considerations on side procedures of (U)HPLC separations will leave us with one quite sobering finding. Although some side actions of a separation can be parallelized to a running separation, a fast separation method alone is by far no guarantee for a high sample throughput and short cycle times. Typically, the most time-burning process is the column equilibration, which in most cases can only be shortened at the expense of reproducibility. If we do not consider injection interlacing steps, then any high-throughput UHPLC analysis is on average extended by 0.5 min for preparing the sample injection and by 1.5 min for column reconditioning, with longer times required easily, depending on sample type, potential wash cycles, and column dimensions. A considerably fast LC method of 2 min then quickly takes double the time, and even an injection preparation parallel to the final phase of a running analysis does not help substantially to shorten the total cycle time. A minimum cycle time of 4–5 min is, therefore, hard to beat, even with the fastest separation programs on the most advanced UHPLC equipment.

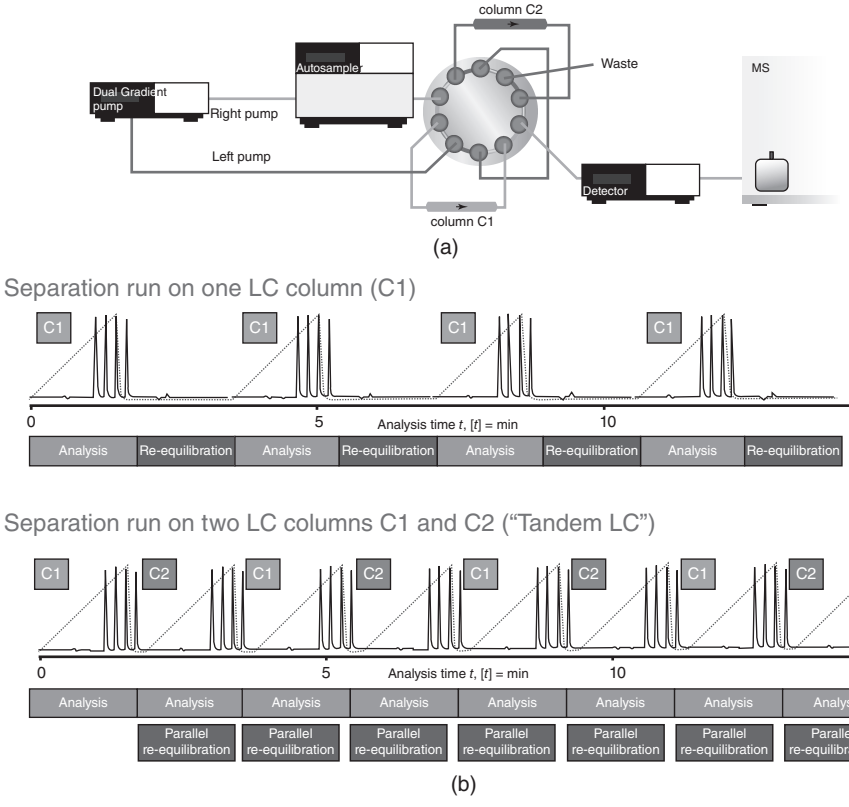


Figure 1.6 Reduction of re-equilibration time and throughput enhancement by using a second separation column and alternating sample injection (tandem LC); (a) flow scheme, (b) injection interlacing.

Case 2 Take-Home Messages

- A rapid separation means much more than an ultra-short (ballistic) gradient program.
- Fast autosamplers help to shorten the delay time prior to the sample injection. Preparing the next injection while the current analysis is still ongoing can help to additionally reduce the cycle time.
- Column re-equilibration is a time-burner, which, however, is hard to avoid, as a thorough column equilibration is mandatory for robust separation results.

Extra-Column Volumes As with UHPLC standalone installations, also LC/MS hyphenated systems are significantly prone to ECV contributions. It is the same rule of thumb that applies here: the maximum ECV between sample introduction and point of detection should not exceed 10–15% of the peak volume of an eluting sample zone. A quick calculation illustrates the situation: a compound

eluting in a 10 s wide peak (baseline width – a level easily undercut by superfast UHPLC separations, which can provide less than sub-2 s baseline widths) at a flow rate of 500 µl/min has a peak volume of 83 µl. This translates in a tolerable ECV of only 8–10 µl. This is even more a challenge in LC/MS, as here the bridge between the UHPLC outlet and the mass spectrometer inlet contributes to the ECV. Thus, this bridging tube ideally would have the smallest volume you can think of, just to minimize unwanted band broadening effects. We can achieve this quite easily by using a capillary of a very small I.D., which should also be as short as possible. However, this capillary cannot be infinitely short – due to the physical dimensions and the geometric arrangement of UHPLC and MS instruments there always will be a certain minimum distance that you will need to bridge. Simultaneously, slim capillaries always generate high system backpressures – just remember Hagen–Poiseuille's law (Eq. 1.2), which describes the capillary pressure as being inversely proportional to the fourth power of the capillary radius:

$$F = \frac{V}{t} = \frac{\Delta p \cdot \pi \cdot r^4}{8 \cdot \eta \cdot L} \tag{1.2}$$

with F = flow rate, V = volume, t = time, Δp = pressure difference, r = capillary radius, η = fluid viscosity, and L = capillary length.

Considering this, we can deduce three general recommendations:

1) *Install your (U)HPLC system in a smart way*

Reducing the pathway length between LC and MS starts already with setting up your UHPLC instrumentation. The conventional LC setup typically follows a top-down path of your mobile phase (Figure 1.7a): with the solvent bottles on top, the stack sequentially contains the degasser, the pump, the autosampler, the column thermostat, and finally the detector(s) downstream. Most of all commercial mass

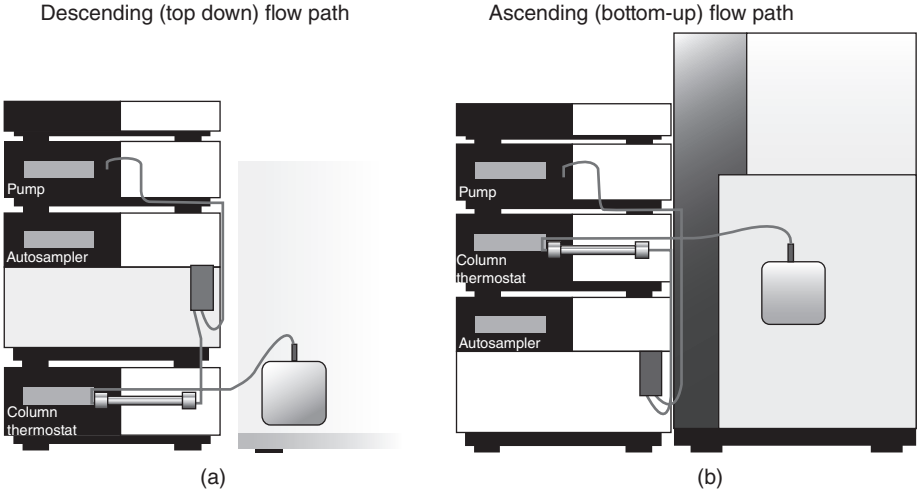


Figure 1.7 Top-down (a) and bottom-up (b) flow path for minimizing the connection tubing length between LC column outlet and MS inlet (ion source).

spectrometers, however, have the ion source inlet placed in a given height above benchtop level, typically between 12 and 25″ (300–600 mm). Hence, a bottom-up installation of the UHPLC flow path would be more appropriate – ideally with the pump and degasser at the bottom, then upwards followed by the autosampler, the column thermostat, and – if required – inline detectors (Figure 1.7b). Most modern compact instruments are already designed like that by default. Modular LC systems can be individually configured by the user, allowing for a reduction of the LC-MS connection capillary length by up to 8–12″ (200–300 mm) in a bottom-up setup compared with a conventional top-down installation. This, for instance, translates into a 2.4 µl void volume saving when using a 100 µm I.D. connection tubing. In some cases, the bottom-up setup may come along with a slightly longer tubing in front of the LC column; however, as already discussed in section "Speed in LC/MS Analysis I: Struggling with the Gradient Delay", this does not noticeably impact the separation quality in case of a gradient separation.

2) *Keep your connection tubing slim and short*

Reducing the internal tubing volume always goes in line with short lengths and small I.D. Thus, the connection tubing between your LC outlet (either the column or, if present, an additional detector, e.g., a DAD) and your MS inlet (the ion source) should have the smallest inner diameter possible, which does not eat up too much of your (U)HPLC system pressure capabilities, and which does not compromise the pressure stability of any part of the flow path prior to the connection line (e.g., UV detection flow cells). As an example, take a connection line of 0.13 mm I.D., having a length of 30″ (750 mm): running this capillary at 25 °C and a flow rate of 500 µl/min would generate a backpressure of moderate 11 bar (160 psi) for a mobile phase with a viscosity of 1.2×10^{-4} Pa·s (which is slightly more than the viscosity maximum of water/acetonitrile mixtures at ambient temperature). However, this capillary would contribute 10 µl of ECV *behind* your LC column, where it is particularly critical. Converting this tubing to 0.10 mm I.D. reduces the ECV contribution to 5.9 µl, but it comes along with a rise in pressure to 31 bar (450 psi); A 0.075 mm I.D. capillary reduces the void volume contribution further down to 3.3 µl, but at the cost of a considerably high backpressure of 97 bar (1410 psi). For further illustration, Table 1.3 summarizes some model calculations for typical LC/MS application conditions. As we can see, a significant speed-up of LC separations at flow rates beyond 1 ml/min is barely possible (leaving out the question if this was useful with respect to the MS detection sensitivity at such high flow rates).

In case you have an additional detector in front of your mass spectrometer, for example, a UV detector, you also have to take care of the detector flow cell pressure limit. Depending on the design principle, the maximum pressure limit of commercial UV flow cells can vary between 870 and 4350 psi (60 and 300 bar). Please be aware that it is not only the MS connection tubing that generates an additional pressure load to your UV flow cell; many mass spectrometers use internal switching valves to introduce calibrant solutions into the MS ion source, which block the flow path completely for a fraction of seconds when they are actuated, thus

Table 1.3 Volumes and backpressure of a 30″/750 mm capillary with different I.D. in the maximum viscosity of water/acetonitrile and water/methanol mixtures.

	Volume	0.13 mm I.D. 10.0 µl	0.10 mm I.D. 5.9 µl	0.075 mm I.D. 3.3 µl
Water/acetonitrile 91/9 v/v, $\eta = 1.06$ cp @ 25 °C	Pressure at $F = 0.5$ ml/min	9.5 bar/138 psi	27 bar/392 psi	85 bar/1233 psi
	Pressure at $F = 1.0$ ml/min	19 bar/276 psi	54 bar/783 psi	171 bar/2480 psi
Water/methanol 40/60 v/v, $\eta = 1.56$ cp @ 25 °C	Pressure at $F = 0.5$ ml/min	14 bar/203 psi	40 bar/580 psi	125 bar/1813 psi
	Pressure at $F = 1.0$ ml/min	28 bar/406 psi	80 bar/1160 psi	251 bar/3640 psi

generating a very short but also very high pressure peak to any technical part in the flow path in front of it. In case you have a more fragile detector cell, you may want to consider either splitting your LC column effluent by a tee piece, or bypassing the MS switching valve and connecting your LC system with the ion source sprayer directly. The former reduces peak efficiencies (by the band-spreading tee piece connection) and sensitivity (only the split fraction of your effluent runs into UV and MS detectors), the latter even tends to improve your peak efficiency in the MS chromatogram, as switching valves in general are plate count killers due to their large bore and groove sizes; however, bridging the internal MS switching valve prevents you from automated recalibration of your mass spectrometer in a sequence run. How critical this is depends on the mass spec type; as already discussed in Section 1.2.1.1, some mass spectrometers need a frequent, if not permanent calibrant infusion, while others do not.

Just as a concluding note – although the tubing I.D. now may be seen as problematic due to its huge impact on the system pressure, it is by far the better optimization parameter to reduce volume contributions. The I.D. goes with the volume by the second power while the length contributes only linearly to it. For comparison reasons, let us consider again our 30″ (750 mm) long capillary of 0.13 mm I.D. (a quite common example for connecting LC with MS), which has an internal volume of approximately 10 µl. To reduce this to the half, you would need to cut the tube down to half the length, so 12.8″ (375 mm) – which would be too short to make your LC/MS connection. Changing to an I.D. of only 0.10 mm (−23%) brings you down to a volume of 5.9 µl, which comes close to the reduction by factor 2, but preserves your original capillary length so that you still have a good chance to be in line with your instrument arrangement. Alternatively, going down to a 0.10 mm I.D. capillary would enable you to make the capillary $(13/10)^2 = 1.7\times$ longer (i.e., 43.4″/1275 mm) but still keeping the same internal volume of 10 µl. We see clearly now: for extra-column and GDV matters, capillary I.D. rules over length.

3) *Take care of your fitting and tubing connection quality*

It is not only the hold-up volume of your tubing that matters. Also, the quality of your fitting system has a large impact on the quality of your LC/MS chromatogram – a factor that is typically underrated in everyday lab life. It is common use still today to connect a UHPLC system with a mass spectrometer by cutting a poly(ether ether ketone) (PEEK) tube of an appropriate length from the bulk and installing it using PEEK fingertight fittings. However, due to improper cutting quality, nonrectangular tubing ends and careless fitting, this introduces a measurable but pointless and avoidable void volume that can be a real efficiency killer. Various dead-volume reducing fitting systems are nowadays commercialized; however, most of them are specially designed and tailored to a respective (U)HPLC equipment and thus not universally applicable. Only four universal UHPLC fitting systems are currently available on the market, not all of them covering true UHPLC pressure loads. These are the Viper™ fingertight fitting technology from Thermo Scientific [78] (maximum pressure of up to 22 000 psi/1500 bar), the A-Line™ fitting design from Agilent Technologies [79] (maximum pressure of up to 18 850 psi/1300 bar), Sure-Fit™ from MicroSolv Technology Corporation [80] (now IDEX; maximum pressure of up to 6000 psi/413 bar), and MarvelX(TM), also from IDEX (up to 19,000 psi/1310 bar) [81]. Figure 1.8 impressively illustrates how using such virtually zero-dead volume connections provide a remarkable gain in plate numbers and resolution. The upper chromatogram (Figure 1.8a) was generated using a standard PEEK capillary of 0.13 mm I.D., cut from the bulk by a standard tube cutter and installed between LC column outlet and MS ion source inlet by regular PEEK fingertight fittings. The lower chromatogram (Figure 1.8b) was run under exactly the same conditions, the only difference being a Viper stainless steel (SST) capillary of identical size and dimension of the PEEK one between column and ion source. The significant rise in chromatographic resolution of 47% from 1.72 to 2.53 clearly lines out how much separation power is wasted in most LC/MS installations simply due to the use of improper tubing and fitting quality.

Case 3 Take-Home Messages

- Keep your eyes on the shortest distance possible between LC outlet and MS inlet already while setting up your UPHPLC system.
- Do not worry about the ECV in front of the LC separation column – it can typically be ignored in gradient elution mode thanks to a sample refocusing effect.
- Focus on the ECV behind the LC column instead: shorter and slimmer tubing always pays off. However, take care of the backpressure generated by very thin capillaries – they eat up pressure reserves of your UHPLC system and could potentially kill your UV flow cell.
- Get rid of any uncontrolled ECV contributions due to improper tubing cuts and connections by using state-of-the-art zero-dead-volume fitting systems.

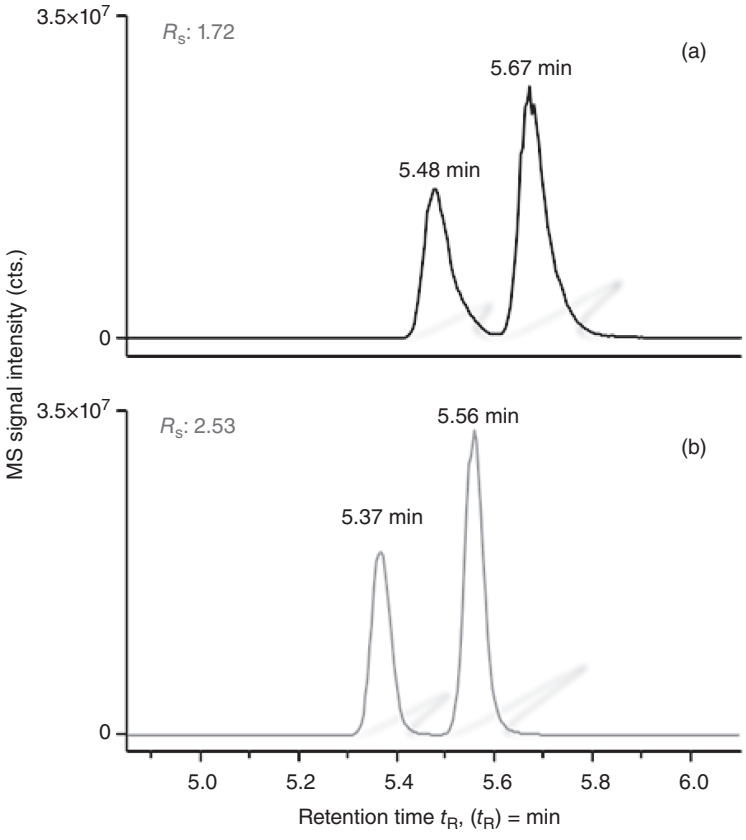


Figure 1.8 LC/MS chromatogram of two isomers, $m/z = 240.10$; (a) PEEK bulk capillary behind the column (0.13 mm I.D.), PEEK fingertight fittings; (b) SST capillary with virtually zero-dead volume connection behind the column (Viper™ fingertight fitting technology, 0.13 mm I.D.).

Data Rates and Cycle Times of Modern Mass Spectrometers It is common sense that an accurate quantitative result can only be generated from the best possible calculation of the *peak area* for your chromatographic peak (and that of reference compounds of course); quantitation based on peak areas beats the peak height determination approach by far with respect to error deviation. Hereby, the higher the number of data points that scan the elution profile of your analyte, the less the error in peak area calculation and in deviation between the experimentally determined and the ideal, theoretical peak profile will be. To ensure an acceptably well-recorded data set, the measured chromatographic peak should at least be described by 25–30 data points. For classical LC detectors, this is no real challenge, as spectroscopic detectors (UV absorption, fluorescence) today provide data acquisition rates of up to 250 Hz, which is more than enough to cope even with ultrafast UHPLC separations and peak widths in the 1-s range.

Mass spectrometers, however, are by far not able to keep pace with the speed performance of state-of-the-art UV detectors. Moreover, data acquisition rates and duty cycles of mass spectrometers behave opposite to the data quality: in most cases, high data rates and short duty cycles come along with poor mass accuracy and reduced resolving power. Especially if the instrument needs to perform complex MS/MS or MS^n experiments, the duty cycles for the individual fragmentation experiments will take so much time that a high chromatographic data acquisition rate simply cannot be realized anymore: the mass spectrometer will then be blind for a new package of the continuously infused ions as long as it processes the experimental steps of the previous set of ions. It is then up to you as the user, to find the ideal balance between the requirements for high-quality *LC/MS chromatograms*, that is, a high data rate for best describing the concentration distribution of an eluting sample zone, and high-resolution *mass spectra* for high-confidence compound confirmation. The exact data acquisition rate of a mass spectrometer hereby depends on many different parameters: instrumental criteria such as the mass analyzer type, the technical features and properties of your particular instrument such as electronics design, processor speed, and so on, and on experimental conditions such as the MS experiment type (whether it is in full scan mode, in SIM or SRM mode, precursor ion scan, etc.), the data acquisition range, or type and number of subsequent fragmentation steps (MS^n, data-dependent or data-independent MS/MS acquisition etc.). Today, TOF mass spectrometers represent the fastest mass analyzers with specified data acquisition rates of up to nominal 200 Hz for MS and 100 Hz for MS/MS runs [75]. This speed sounds very impressive, but it should be mentioned that this high speed does not allow to simultaneously achieve highest resolving power and spectrum quality. For comparison, triple quadrupole mass spectrometers that are the most widely used MS types for routine quantitation offer typical data acquisition rates of 5–15 Hz. This can already be challenged by a well-optimized conventional HPLC separation – to meet the requirements of ultrafast UHPLC separations with ballistic gradients, this will definitely be too slow. For a more detailed discussion on the selection of appropriate data rates, refer to Chapter 2.

Complementary Information by Additional Detectors or Mass Spectrometry Will Not Save the World It is a well-known saying that mass spectrometry is one of the most powerful analytical tools the world has ever seen. Without a shadow of a doubt, the sheer amount, the detail degree, and the accuracy of analytical information provided by mass spectrometers is very impressive; however, they cannot solve the impossible, and performing miracles beyond common sense is also not their business. Here is a small collection of the most widespread hypes and (partially) wrong assessments on mass spectrometers:

A Mass Spec Is a Universal Detector: This is a frequently quoted claim, which, however, is not getting right by frequent repetition. The advocates of this phrase typically compare MS with UV detection, referring to the fact that spectroscopic detection could only measure analytes having suitable chromophores that

interact with electromagnetic waves of a certain energy (represented by the wavelength). This is perfectly right, but unfortunately it is only an indicator for the selectivity of UV absorbance detection, but not for the pretended universality of MS detection. Indeed, it ignores the fact that also the detectability in a mass spectrometer is analyte-dependent, because it relates to the ionizability of your compound of interest; this, however, is not only related to the amount of ionizing energy present in the ion source, but also to analyte-specific properties. Molecules that do not have a considerable gas phase proton affinity will lead to a very poor ion yield in ESI or APCI mode, resulting in only a low amount of detectable molecular ions. Hence, every analyte species has its own individual mass spectrometric response factor, which indeed might be too low for a proper MS detection, depending on the selected ionization principle. Simply said: ESI and APCI are selective toward molecules with a certain gas phase acidity or basicity. Mass spectrometers are highly flexible in their wide application range and can easily be adapted to analyte requirements by a simple change of the ionization mode – but they are far away from being a universal detector.

It should be mentioned here that in many conversations and also in some literature there is no clear distinction between a *universal* and a *uniform* detection. The latter one describes the requirement of providing a homogeneous, identical response factor for all analytes of interest, independent from their molecular properties. This not necessarily has to come along with universal detection, but in real life it is an extended feature of (virtually) universal detectors. However, if a mass spectrometer does not detect universally by definition, there is even less of an argument for a mass spec being a uniform detector, due to the different ionizability of the analytes. This implies that for all quantitation experiments, the mass spectrometer must be calibrated for each individual analyte – which comes along with a significant experimental effort. True universal detectors in contrast (or technologies that come close to this ideal), such as charged aerosol detectors (CAD) or evaporative light scattering detectors (ELSD), can massively reduce (for exact quantitation) or even virtually eliminate the calibration efforts (for semiquantitative results and/or with constant matrix content).

There's No Detector Which Is More Sensitive than a Mass Spec.: This phrase touches the same misapprehension as the previous one. Sensitivity and the LoD and LoQ in mass spectrometry are not by default superior to any other detector. Under favorable conditions, like high ion formation yield and good ion transmission through the mass analyzer to the mass detector, mass spectrometers are indeed very powerful, allowing LoQs down to a femto- or even attomol level. However, in case of poorly ionizable analytes, an inappropriate ionization principle and/or perhaps not the most sensitive MS instrument design, there may be other detection principles that are clearly in favor, for instance electrochemical or fluorescence detection.

Identify All Your Analytes with 100% Certainty Using a Mass Spec.: Also this claim cannot be confirmed without limitations. Whatever you could do with your molecular ions in a modern mass spectrometer, such as performing sophisticated

gas phase experiments – at the end of the day, a mass spectrometer is "merely" a highly accurate kind of balance to determine molecular masses. However, identical molecular masses by far do not equal identical molecular structures and appearances. Even the most accurate molecular mass determination will fail in distinguishing isobaric compounds entering the mass spectrometer at the same time, without further discrimination. Any kind of isobaric compounds – *E*/*Z* isomers, diastereomers, enantiomers, and so on – cannot be differentiated by an MS experiment in the first way, as they all have the same molecular mass. As soon as isobaric species show a different fragmentation behavior, you will have a certain chance to separately identify them based on their fragmentation pattern in an MS/MS spectrum; however, this is unfortunately not a given in many cases.

All these aspects clearly show that other, more classical detection principles are by far not obsolete only because you have a mass spectrometer in your lab. Especially, when it comes to the structure elucidation of small molecules, spectroscopy (UV absorption, fluorescence) or electrochemistry provides valuable and complementary information helping to interpret your MS results (with NMR being the gold-standard of course, but LC-NMR hyphenation is by far more complex and less widespread than most other detection principles). One example to showcase the issue with isobaric compounds: many isomers differ significantly in their UV absorption spectrum; and due to the unique capability of reversed-phase (RP) chromatography to provide a structural recognition mechanism, they will also show different retention times. This is illustrated in Figure 1.9, which shows a reaction control analysis by LC-UV-MS to monitor the progress and yield for an *N*-aryl coupling reaction; unfortunately, the educt is not pure but is contaminated with a certain amount of the competing isomer – which could potentially lead also to an *E*/*Z* mix of product molecules. The coupling reaction indeed is stereoselective as expected (only one product isomer can be detected); however, based on the LC/MS chromatograms alone, there is no way to correctly assign the two educt peaks to the related *E* and *Z* isomer – the extracted ion chromatogram (EIC) for the educt mass just shows two peaks belonging to the two educt species, without any further indication of which one is which. However, as the *E* and *Z* educt isomers show significantly different UV spectra (not shown) and RP chromatography provides you with an excellent separation of the two species, a correct peak assignment based on the diode array detection spectra can be achieved seamlessly.

Whenever you plan to combine a mass spectrometer with a second detector, it is this additional detection principle that tells you how to technically realize the hyphenation. Do not forget – the mass spectrometer always eliminates your analyte while measuring and detecting it, so it must be the last detector in your instrument arrangement. If you want to add a nondestructive detector to your system – all spectroscopic detectors are of that kind – you can simply connect this in line with your LC column upfront and the mass spectrometer behind. One thing to take care of is the additional volume of the detector flow cell, which in most cases also adds a measurable contribution to band broadening. Using UV

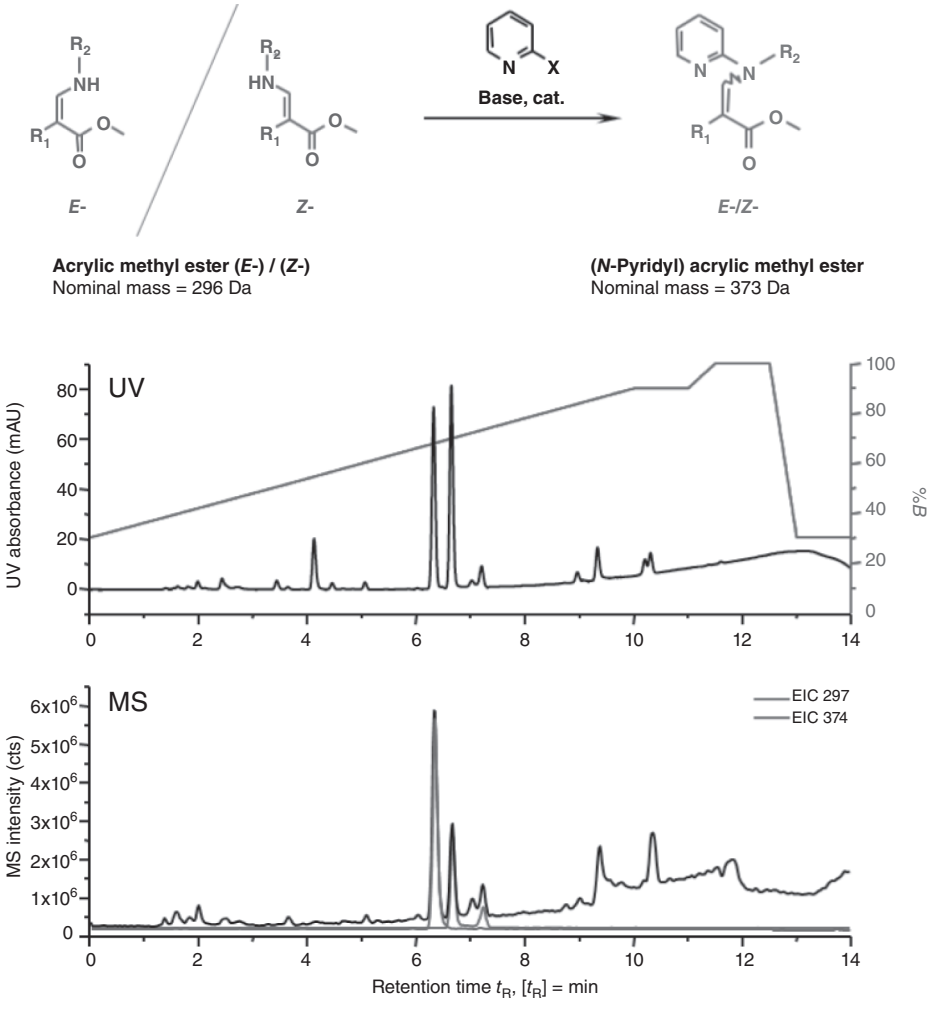


Figure 1.9 Distinction of isobaric compounds by RP chromatography and UV detection for a reaction control analysis (*N*-arylation of an *E*/*Z* acrylic ester mixture).

detectors, you would thus ideally look for a flow cell with a very small internal volume at maximum light path length. Typical UV flow cells of conventional design are commercially available with volumes down to 2–5 µl. A very popular topic these days are flow cell designs featuring a capillary-based flow cuvette, glass-fiber optics, and internal total reflection, enabling very long light paths of 10 up to 60 mm, combined with very low internal volumes, which allow for remarkably low levels of detection and a superb sensitivity. Unfortunately, these flow cell designs have two inherent drawbacks: next to their high price, the term *high sensitivity* also applies to their lower mechanical ruggedness. To some degree, all these

high-sensitivity cells have a pressure limitation below 1450 psi/100 bar, which is considerably lower than for conventional UV flow cells. As long as the UV detector is the last in your row, this does not really matter, but in combination with a mass spectrometer (or, for instance, a fraction collector) it does. Another alternative in this case would be specifically optimized capillary flow cells. They are made from fused-silica capillaries and can be used and handled like any other conventional flow cell; they combine excellent pressure resistance (up to 4350 psi/300 bar) with impressing low internal volumes of less than 50 nl. However, these cell types are highly limited in their linear range, which does not recommend them for quantitative analyses, but makes them the ideal UV monitor cell with no measurable band-broadening impact on a classical UHPLC separation on 2.1 mm I.D. columns.

By contrast, destructive detection principles like nebulizer-based detectors (CAD, ELSD, etc.) must be connected parallel to the mass spectrometer using a tee piece. This may sound clumsy, but it is not totally disadvantageous. Depending on the LC method settings, apost-column split may be a good idea anyway – it would allow you to run your LC separation with the best-suited linear velocity (in the van Deemter minimum of your column or beyond) and still to reduce the flow rate entering the MS ion source. A potential drawback of the split flow approach would be the addition of another ECV, potentially affecting your chromatographic efficiency and resolution. But in turn you would end up with a very versatile and powerful analysis tool: combining a uniform detection principle such as CAD with mass spectrometry accelerates screening experiments massively. One single chromatographic run in such a setup will give you a very good (semi)quantitative result from one of the most unspecific detectors commercially available, while a parallel-running HR/AM mass spectrometer gives you excellent qualitative data for (virtually) unambiguous compound identification. A very interesting extension in this context can be offered by electrochemical detectors (ECD). By making use of the redox activity of analytes to generate a detector signal, they will alter the analyte species; a MS in series behind will thus no longer detect the molecular ion mass of the initial species, but of the oxidized or reduced one. If you needed the unchanged mass of the original analyte, you would then add the ECD in a parallel split. By plugging the ECD in line with the mass spectrometer, however, you will get the highly interesting option to enhance the detection sensitivity of your MS by electrochemically converting your analytes into oxidized or reduced species that may show a much better signal response than the original molecule. You can even use this approach for bioanalytical applications, for instance, by electrochemically mimicking certain metabolism processes and to investigate them immediately, on the fly so to speak, in the mass spectrometer.

Case 4 Take-Home Messages

- Mass spectrometers are not universal detectors.
- Mass spectrometers detect highly selectively, depending on their operation settings, but not specifically.
- For the unambiguous compound structure confirmation, you will always need (at least) one additional structure elucidation method (e.g., NMR spectroscopy) next to your MS(/MS) findings.
- In all cases where molecules cannot be distinguished based on their molecular mass within the experimental error (isobaric compounds), additional and complementary detection principles are imperative. The related detector modules can be added either serially or in parallel by splitting the LC column effluent, depending on the detection principle.

1.2.2 When LC Methods and MS Conditions Meet Each Other

When LC meets mass spectrometry, two very different worlds with highly contrary physical requirements need to come together. While an HPLC separation works against ambient pressure at the outlet end, a mass spectrometer always asks for a high-quality vacuum to operate. The interface between LC and MS, the ion source, must therefore handle multiple tasks simultaneously: transferring the dissolved analytes into the gas phase, separating the analytes from the residual mobile phase (typically done by gas phase transfer), controlled ionization of the analyte molecules, and a focused analyte ion transfer into the evacuated mass analyzer. None of these jobs is a simple one – just take the mobile phase removal: as we know, the molar volume of a gaseous compound is 22.4 l under normal and 24.5 l under standard conditions. Hence, water at a flow rate of 1 ml/min, equaling 1/18 mol/min, forms 1.2 l of vapor every minute, which needs to be completely removed from the analytes and drained out of the ion source. Therefore, the LC separation needs to meet certain limiting requirements to ensure a smooth signal generation in the mass spectrometer.

1.2.2.1 Flow Rate and Principle of Ion Formation

Let me open this section with a short remark on the term *sensitivity*. In the first instance, "sensitivity" is defined as the slope of the response function that describes the signal change depending on a change in analyte amount or concentration. The slope, also known as response factor, always has a physical

dimension (signal unity per concentration measure), which makes it impossible to directly compare response factors of different detection principles. However, the sensitivity is always linked to the ratio of signal intensity to baseline noise – a dimensionless measure, which is also used to determine LoD and LoQ (and which allows a comparison between different detection methods). The following discussion covers both of these interpretations.

It is already the selected ionization principle that tells you about the maximum LC flow rate you should confront your mass spectrometer with. ESI, which is applied in about 82% of all published online-LC/MS hyphenations (the rest is shared between APCI with 16% and APPI and others with 2%; LC-MALDI-MS in contrast is a classical offline hyphenation example) [75], enables the use of 50 – 300 µl/min flow rates at best sensitivity if assisted by a pneumatic nebulizer gas [81]. To some degree, all commercial ESI interfaces (excluding nanospray sources) can be operated at much higher flow rates of up to 1 ml/min and more. While being a concentration-sensitive process, ESI by theory is barely affected by the flow rate; the peak height should not change significantly with the LC flow rate. Indeed, there are many literature examples demonstrating that the sensitivity of ESI methods suffers only if the excess of mobile phase cannot be removed effectively anymore. This, however, can happen at LC flow rates beyond 1 ml/min, depending on the ESI interface design and the efficiency of the source heating or the supporting nebulizer [82]. In reality, experimental conditions such as composition and mixing change of the mobile phase over time can lower the sensitivity already at flow rates higher than 300 – 500 µl/min. It is hard to predict the extent of this reduction as there is no mathematical model or a rule of thumb for this; so it is highly recommendable to monitor the signal intensity and the signal/noise ratio for your target analytes at different flow rates by a flow injection analysis (FIA). Depending on the solvent removal capacity of the ESI interface, the maximum sensitivity might be reached at flow rates that are lower than the van Deemter minimum of the LC column packing material. Then, it is a case-by-case decision where to set the priority, on highest detection sensitivity or best chromatographic efficiency.

APCI tolerates much higher flow rates than ESI, which lies in the nature of the process – it simply needs a minimum amount of solvent vapor to create the reagent gas that is responsible for the analyte ionization. APCI is a mass-sensitive process [83], which benefits from higher flow rates because more analyte molecules per time enter the APCI interface, thus leading to increased peak heights at higher flow rates (the exception proves the rule [84]). The operation range of APCI starts at 150 – 200 µl/min and ends at maximum 2 ml/min, with a sensitivity loss being observed also here at very high flow rates, depending on the interface design and the evaporation capacity. Similar to ESI, monitoring the sensitivity in dependency of the flow rate should be a no-brainer to determine the ideal LC/MS flow rate during the MS method development. Table 1.4 summarizes the usable and the most effective working ranges of ESI and APCI.

Hence, APCI seems to be a very suitable interface principle, particularly for fast UHPLC-MS separations. It should also be mentioned that APCI is less prone to matrix effects in many applications, thus having the tendency of being more

Table 1.4 Applicable and ideal working ranges for selected ionization processes.

	Applicable working range	Ideal working range
Nano-ESI source (without nebulizer gas)	<5 µl/min	20 – 800 nl/min
Standard ESI source (with nebulizing support)	0.01 – 1.5 ml/min	0.05 – 0.3 ml/min
APCI source	0.2 – 2 ml/min	0.3 – 1 ml/min

robust and accurate than ESI (refer also to section "Gas Phase Adducts"). However, the user is not free in his or her choice, as the analyte properties dictate which ionization principle has to be applied. Due to the analyte polarity, ESI is the default choice in most cases. However, a dedicated LC column hardware can comply very well with the low flow rates ideal for ESI: columns of 2.1 or 1 mm inner diameter allow operating even UHPLC stationary phase materials with average particle diameters below 2 µm at optimum linear velocities, as this translates in still very low volume flows in the microliter per minute range at those small column I.D. If the sensitivity loss with ESI at ideal chromatographic linear velocity was still too high, a post-column split of the LC effluent could be a good way out of this dilemma. Such a split is easily realized by a tee piece and two restriction capillaries – their dimensions will determine the split ratio between the primary flow to the MS and the bypass to the waste. As an extension, the split bypass does not mandatorily have to go to the waste: it can also be used for a second, ideally mass-sensitive detector. Just think of the combination of a mass spectrometer and a nebulizer-based detector such as ELSD or CAD, characterized by a virtually uniform, analyte-independent response, which would give you a very powerful tool for the identification and parallel (semi)quantitation even of unknown compounds. Nevertheless, such a split always bears also the risk for band-broadening void volumes, so great care must be taken while selecting the different pieces and assembling the split construction.

1.2.2.2 Mobile Phase Composition

MS compatibility of the mobile phase means that *all* ingredients of the eluent have to be volatile. To some degree, all solvents used in RP chromatography comply with this rule; water, which is the chromatography liquid with the highest evaporation enthalpy, is very well compatible with ESI and APCI processes – even more, a minimum amount of water is vital for an acceptable ionization yield. Organic solvents enhance the spray drying not only due to their higher vapor pressure but also by reducing the surface tension of the solvent droplets in the electrospray, which facilitates the evaporation of residual solvent molecules. As a practical consequence, higher organic content leads to a better spray stability and increased signal/noise ratio – which can also be observed in any RP gradient run; in ESI, the signal intensity typically goes linearly with the increase in organic content in the

mobile phase at up to 80% [81]. The trend behavior can significantly differ between pure solvents and solvent mixtures – for a more detailed discussion refer to the literature [85].

Volatility, however, must also be a given for all kinds of additives to the mobile phase. All modifiers forming nonvolatile salts or precipitates lead to a massively enhanced suppression of ion formation in the ion source (*ion suppression*) and to a rapid and tough contamination of the ion source with salt crusts. Next to the frequent cleaning efforts, this leads inevitably to a pronounced sensitivity loss in your MS chromatogram, although many major instrument vendors claim the opposite. As a consequence, most of the classical buffering agents, acids, bases, and additives well-known in LC standalone such as phosphate or borate buffers, and more generally sodium salts have to be avoided in combination with LC/MS. Instead, various (semi-)volatile organic acids, bases and their respective ammonium salts are viable alternatives. For acidic pH, formic acid (FA), acetic acid, and trifluoroacetic acid (TFA) are most popular, while aqueous ammonia solutions or alkyl amines, for example, triethyl amine, cover the alkaline range. If buffer capacity is needed, ammonium salts such as ammonium formate, acetate, or bicarbonate are of first choice. Also, oxidizing agents are not appropriate for LC/MS eluents: it is known that chloride ions not only promote ion suppression, but the electrospray can oxidize them into chlorine, which chemically modifies your analyte and over time also attacks your ion source hardware such as the spray needle [86]. Volatile ionic detergents, however, are also not a good choice, as they can deposit on the surfaces of the ion optics and the mass analyzer when entering the mass spectrometer; this can lead to electrical discharges and instrument malfunctions over time.

A topic frequently and controversially discussed in literature should briefly be mentioned here, which is the pros and cons of the use of FA and TFA as modifiers in LC/MS applications. These moderate organic acids generate a pH of 2–3, depending on the concentration, while still being MS-compatible as they do not form any nonvolatile precipitates in the ion source. Simultaneously, their anions – formate and triflate – are also ion-pairing reagents and thus actively controlling the retention mechanism of many analytes. Hence, cationic analytes potentially show a higher retention on an RP stationary phase. In many cases, also an improved peak shape with lower asymmetry can be observed. However, this effect, which is much appreciated in liquid chromatography, has its drawback in the MS ion source, as it compromises or even suppresses the formation of ions in the gas phase – a classical conflict of interests for the LC/MS analyst. TFA hereby is a much more effective ion-pairing reagent than FA. This leads to a better chromatographic elution behavior with enhanced retention and reduced peak asymmetry, but it also comes at the price of a noticeably worse signal/noise ratio in the MS chromatogram due to stronger suppression of ion formation in the ion source. When analyzing small molecules, the retention-enhancing effect of TFA is not much pronounced, but you clearly will observe ion suppression. Therefore, FA is more appropriate for the LC/MS analysis of small molecules. A classical tradeoff is the analysis of larger biomolecules, such as peptides or proteins,

which need to be detected in very low concentrations. For best sensitivity, FA would also here represent the modifier of choice, but TFA can significantly improve the chromatographic performance. For bioanalytical questions, which frequently are realized including a trap column in an online enrichment setup, it is, therefore, recommendable to use TFA in the trap column flow path, but change to FA in the analytical separation; in some cases, the addition of TFA also in the analytical flow path may be inevitable, though. Figure 1.10 illustrates nicely the retention-enhancing effect of TFA.

1.2.3
Quality of Your Mass Spectra and LC/MS Chromatograms

The previously discussed considerations cover very fundamental aspects of an LC/MS method setup; they are discussed typically once, at the beginning of your work, as they deal with generic questions such as "which instrument should I use" or "how should my LC/MS method look like." Once decided, these things do not change significantly across the lifetime of a method. But there are many minor

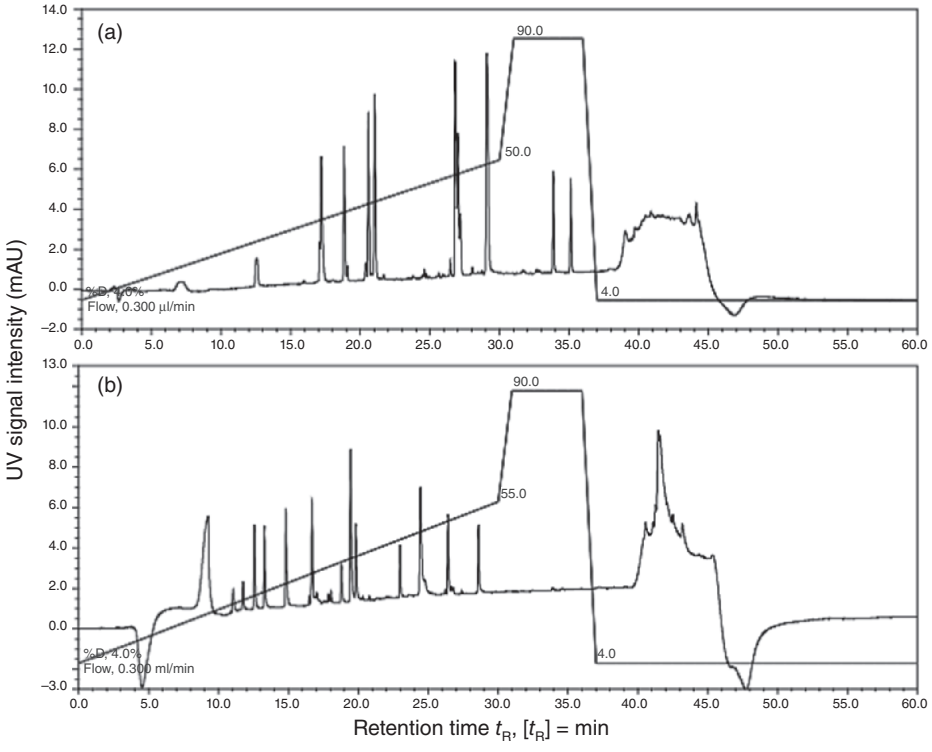


Figure 1.10 Separation of a cytochrome C digest by adding 0.05% TFA (a) or 0.1% FA (b) under identical chromatographic conditions.

or major deviations from the routine happening in the lab every day. Surprises and pitfalls are many, from wrong mass assignments or unknown mass signals, noisy MS spectra to a totally empty mass signal and sheer baseline noise. This final section should help to showcase very common error symptoms of LC/MS lab life and to point out ways how to tackle them.

1.2.3.1 No Signal at All

The most striking problem while running a sample analysis is described very quickly: after thoroughly developing a robust and sensitive LC/MS analysis method, you start an injection and you see-nothing. Obviously, the root cause for this "nothing" can be either the LC or the MS part of your hyphenated LC/MS system, with the worst case being that both chromatography and mass spectrometry do not run as expected. The hard truth is that in most of these cases, the instrument hardware is operating properly – none of the diagnostic tools for the devices report any error for the electric and electronic instrument subassemblies. Most of the time, it is an inadequate ion formation that is responsible for weak or no MS signals, so the system struggles somewhere with the ion source, the ion transfer to the vacuum section of the MS, or with the sample introduction. Technical defects behind the ion source, be it in the ion optics, the mass analyzer, or the mass detector, are observed much less frequently.

To pin down the error source, it is very helpful to check first the signal intensity and the noise level of the baseline both in the LC/MS chromatogram and in the mass spectra via the online view of the instrument control software. A very low signal intensity of only several hundreds of ion counts and a noise pattern that looks much more like an erratic flaring of signal spikes than a continuous base level signal are strong indicators that virtually no ions at all are reaching the mass analyzer or the mass detector. The core reason for this is typically a quite rough mechanical or electrical defect either in the LC or the ion source sprayer assembly. To further troubleshoot, the LC part of your system, a UV detector is of invaluable help – no other detection principle is even par with UV with respect to ruggedness. Nowadays, dedicated UV monitor flow cells of only a few dozens of nanoliters internal volume allow even for a permanent monitoring of your LC/MS separation all the time as they do not contribute measurably to band broadening and thus reduced peak efficiency. This allows for a rapid and doubtless verification of your chromatographic separation running properly. Having confirmed that chromatography separates your analytes and transports them toward the outlet of the LC/MS connection capillary accordingly, there must be something wrong within the MS ion source. For instance, a deformed or even broken spray capillary or needle prevents effectively the LC effluent from entering the MS ion source; hence, no stable nebulizing spray can be created. Very popular here is a spray needle tip that is cracked, resulting from a short touch on the lab bench or during a careless insertion into the source assembly. ESI and APCI ion sources that apply the HV for the electrospray or ion formation to the spray needle (such as Sciex and Thermo Scientific devices) instead of the MS vacuum inlet

(Bruker Daltonics, Agilent Technologies) can additionally suffer from a damage in the electrical wiring or plug connections due to improper handling.

With a chromatography and spray unit assembly being intact and a baseline noise that indicates a reasonably stable spray being formed, obviously your system generates ions properly, but for whatever reason a too little amount of ions truly reaches the mass analyzer. The ion current measured along the ion transfer capillary in the evacuated part of your mass spectrometer is a good indicator for an acceptably high ion transfer yield. In the case of this being very low, your first step of action should be a thorough clean-up of the ion source including the ion transfer capillary. Analyte or dirt deposits on the various surfaces, which cannot even be seen by your eyes, can suppress ion formation and need to be removed by cleaning. It is highly recommendable to check the state of the spray needle, particularly the tip, with a magnifier or a suitable microscope. If the needle tip is bent or cracked, you will need to replace it. Depending on the sprayer assembly design and thus MS vendor, also the alignment and positioning of the spray needle in the sprayer assembly should be checked; a re-adjustment of the needle alignment can be very helpful, as the extent of the tip protrusion out of the sprayer assembly can change the detection sensitivity by several orders of magnitude.

1.2.3.2 Inappropriate Ion Source Settings and their Impact on the Chromatogram

The baseline quality of our LC/MS chromatogram is a good indicator if the processes within your ion source are running seamlessly; same for the base signals in every individual mass spectrum. Typical error patterns can mostly be divided into two categories: increased baseline noise and poor baseline stability. Both phenomena suggest that something is going wrong with the selective creation of analyte ions or the continuous removal of residual solvent, for instance due to spray instability. An increased baseline noise is mostly a clear sign for dirt into the MS ion source, as it is the result of too many different ions being created over a wide m/z range and entering the mass spectrometer simultaneously. The reasons for this so-called *chemical noise* can be manifold. The first thing to check would be the cleanliness of the ion source and when it has been cleaned up most recently. Any residues on the spray needle tip, on the internal surfaces of the ion source assembly, on the orifice and metal plates of the vacuum inlet, on the ion transfer capillary or the first stages of ion lenses are very good candidates for increased baseline noise. Very obvious sources for these chemical contaminations are not only sample or matrix components but also wanted or unwanted parts of your mobile phase, and the dry gas or nebulizer gas of the ion source. The LC solvents used to compose your mobile phase should, therefore, always be of LC/MS purity grade (labeled "LC-MS grade", "ULC/MS", etc., depending on the supplier). The very popular "gradient grade" purity solvents, however, are merely optimized for a minimal amount of UV-absorbing impurities and, therefore, are typically not pure enough for LC/MS applications.

The gas used to dry and/or to nebulize the LC effluent – typically nitrogen – can also introduce contaminations into the ion source and thus reduce

your signal quality. The gas purity level should be at least 99.0% or better 99.5% and higher. Depending on the nitrogen origin, the types of contaminants can vary. Many mass spectrometers have a very high gas consumption of up to 10 – 25 l/min, especially when combined with analytical-scale LC flow rates of several hundreds of microliters per minutes. This locks out the nitrogen supply from gas cylinders – although this ensures a very high gas purity, the average gas consumption of a mass spectrometer would typically empty these cylinders every 1 – 2 days, which is not very economic and not convenient either. Higher amounts of nitrogen can only reasonably be provided by nitrogen generators or a nitrogen supply line in your lab based on liquid nitrogen evaporation. The latter typically ensures the highest gas purity grade. Nitrogen generators, in contrast, are fed with pressurized ambient air and then the oxygen removed by a membrane separator. The pressurized air, however, is provided by a compressor and, therefore, frequently contains residual oil mist and other hydrocarbons diffusing out of the compressor hardware. These contaminants must be effectively eliminated from the nitrogen stream by gas filters based on activated carbon filter assemblies or other adsorbents. If you miss such a filter or the filter is fully loaded and needs to be replaced, you will observe a significant rise in the baseline noise, too.

Next to these "real" contaminations originating from unintended chemicals, gas phase aggregates or *clusters* built from residual solvent molecules and charge carriers can be formed under nonoptimized ion source conditions. These clusters can in summary be heavy enough to create signals within the m/z detection range of your MS experiment and thus permanently contribute to the baseline noise. A thorough optimization of the ion source parameter settings can effectively suppress this cluster formation. Figure 1.11 illustrates this by depicting the MS signal intensity of a target analyte (Astemizol, m/z 459.3) introduced by FIA under varying dry gas temperatures. In Figure 1.11a, we see that the individual analyte signal intensity in the EIC remains nearly constant with rising dry gas temperature, so the analyte ion yield does not really change with higher temperatures. Figure 1.11b reveals, however, that the overall noise level in the TIC significantly varies with the drying temperature, which is a clear sign for a more efficient gas phase aggregate destruction (*declustering*).

Finally, an increased baseline noise can also be caused by too high of an electrospray voltage (called ESI voltage in the following). In extreme cases, you can even see this with the naked eye as a pale blue glow discharge on the ESI needle tip. This glow discharge is facilitated by too many charged species in your mobile phase, for instance, by using a highly concentrated buffer salt, acid, or base; next to a disrupting ion beam in your MS source, this can even lead to a voltage flashover in parts of your ion optics. If possible, you should then reduce the ESI voltage, the buffer concentration in your eluent, or both.

Baseline stability issues, in particular spikes or spontaneous drops, typically indicate an improper nebulizing or an unstable electrospray. A poor nebulizer gas flow or pressure rate as well as a nonideal ESI voltage then lead to the formation of larger liquid droplets or clusters in the ion source; these droplets tearing down from the ESI needle will lead to negative drops in your baseline, while a

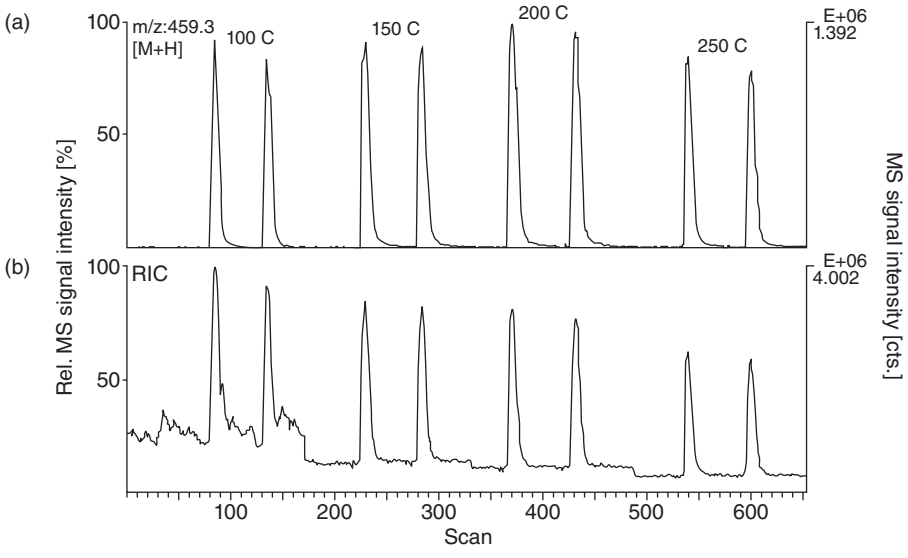


Figure 1.11 Influence of the dry gas temperature on MS signal quality using FIA of Astemizol in 10 mM aqueous ammonium acetate/methanol 20/80 (v/v) at 50 µl/min on a Triple-Quad instrument; (a) extracted ion chromatogram of Astemizol ($[M+H]^+$), *m/z* 459.3; (b) reconstructed total ion chromatogram.

droplet burst releases many ions from this droplet simultaneously and thus generates characteristic positive spikes.

1.2.3.3 **Ion Suppression**

Whenever possible, the LC/MS method development is done with high-purity reference compounds. Real samples, however, can host significant amounts of impurities and matrix components; those can lead to a substantial signal intensity reduction for your analytes at the same identical concentration as in your purified method development standard solutions. This is a very frequent problem in matrix-rich sample analyses such as food, blood and plasma, or cell tissue. The origin of this deviation in signal intensity is the co-elution of the target analyte with other compounds that interfere with each other during the ionization process and thus affect the ion yield for your analyte of interest. Depending on the mechanisms behind, either a signal enhancement or a signal reduction could be observed, the latter being called *ion suppression*, which is already discussed in Section 1.1. The reasons for a signal-enhancing or signal-reducing effect are manifold. An in-depth discussion would exceed the scope of this section, so I refer to the literature instead [87]. Signal suppression can be observed both with ESI and APCI interfaces, for positive as well as negative polarity. However, there are many examples indicating that APCI is less prone to ion suppression than ESI, and negative polarity seems to be less affected than positive. We already learned about how to qualitatively determine the signal enhancement or suppression effect

using a *post-column infusion* setup (see Section 1.1). A quantitative assessment of matrix effects requires a comparative analysis of a spiked, matrix-free sample and a matrix-containing sample. A comparison of peak areas from both experiments will tell you then about the extent of a signal change by matrix effects. It is explicitly this signal intensity change caused by matrix effects that passionately pleas for a high-quality LC sample separation as an essential requirement for a robust and reliable quantitation with mass spectrometry. Infusing an unknown and nondiscriminated bulk solution of your sample into a mass spectrometer will always have the potential for many disturbing interactions of the analytes with each other during the ion formation process; as a result, you would barely be able to assess whether you truly see every analyte in your sample, not to speak of a reliable analyte quantitation.

1.2.3.4 Unknown Mass Signals in the Mass Spectrum

Many LC/MS users are permanently facing the challenge that they cannot plausibly assign all signals in a mass spectrum to a given target analyte. Either the measured mass signals differ from what is expected, or the spectrum shows more m/z values than predicted. In the following, we discuss a selection of root causes being responsible for this mismatch between experiment and expectation. However, it would be beyond the scope of this book to talk comprehensively about virtually all aspects of observing unknown mass signals; therefore, I refer also to MS-specific literature for further reading [76, 88, 89].

Gas Phase Adducts The most frequent reason by far for unknown mass spectrum peaks is the creation of adducts between the analyte molecular ion and other low-molecular-weight ions and/or neutral chemical entities in the gas phase. Hereby, the type and extent of adduct formation varies substantially with the ionization principle, the ion source parameter settings, the analyte properties, and the quality of the mobile phase. However, gas phase adduct formation or *clustering* is not disadvantageous by default; it is even imperative for the conversion of a neutral molecular species into a charged one with ESI and APCI, as adding a proton to (in positive polarity mode) or subtracting a proton from (in negative polarity) an analyte of the molecular mass M converts it into the charged state of $[M+H]^+$ and $[M–H]^–$, respectively. Consequently, the measured m/z ratio differs from the theoretical one of the neutral species by the amount of one proton mass. But next to this fundamental prerequisite for the mass detection, many other charged adduct species can be observed in reality. The virtually ubiquitous sodium and potassium cations leaching from the glass surfaces of the solvent bottles, for instance, frequently lead to the respective adducts $[M+Na]^+$ and $[M+K]^+$. The longer the shelf life of your solvents, the more these sodium and potassium clusters are even in favor compared with the proton adduct – shifts in the adduct ratio between proton and alkali metal-based ionic species can, therefore, even be used as a rough estimate of the solvent age in your LC/MS system. With the sodium or potassium adduct becoming the most prominent m/z signal in your spectrum over time, it is high time to

prepare a fresh lot of mobile phase for your UHPLC separation. Due to the well-known chemical similarity between the alkali metal ions and the ammonium ion, the use of ammonium salts as buffering agents in LC/MS applications will lead to the analog creation of the ammonium adduct $[M + NH_4]^+$ instead. Next to those species, also higher aggregates involving three or more – mostly neutral – molecules can be observed; depending on the solvent evaporation efficiency, excessive solvent molecules can then cluster with the analyte and the charge carrier, resulting, for example, in $[M + H_2O + H]^+$ or $[M + CH_3CN + H]^+$. Even clusters of multiple analyte molecules sharing one proton or sodium cation as $[2M + H]^+$ or $[2M + Na]^+$ can often be detected. All these adducts reveal very characteristic mass differences in comparison to the simple proton adduct. Table 1.5 lists the most common gas phase adducts with their respective nominal mass difference to the singly protonated ($[M + H]^+$) or deprotonated ($[M\text{-}H]^-$) reference. More in-depth information on that matter can also be found in the literature [90].

In general, the trend to form those adducts can be controlled quite effectively via the ion source parameters. An appropriate setting for the drying conditions of the source (such as dry gas temperature, nebulizer gas pressure) allows the declustering of higher aggregates into less complex ones. Also, APCI is typically less prone to higher aggregate formation with, for example, alkali metals, as the charge transfer to the analyte molecule happens after the evaporation, that is, entirely in the gas phase; In ESI, in contrast, the charge transfer takes place parallel to the gas phase transfer, so while the analyte still partially is in the liquid phase. For the same reason, APCI is in many cases also less affected by matrix effects, depending of course on the application. As the cluster formation is also influenced by type

Table 1.5 Common gas phase adducts at positive (left) and negative (right) polarity.

Positive polarity		Negative polarity	
Gas phase adduct	**Nominal mass difference (ΔDa)**	**Gas phase adduct**	**Nominal mass difference (ΔDa)**
$[M + NH_4]^+$	+17	$[M - H + H_2O]^-$	+18
$[M + H_2O + H]^+$	+18	$[M - H + CH_3OH]^-$	+32
$[M + Na]^+$	+22	$[M + Cl]^-$	+36
$[M + CH_3OH + H]^+$	+32	$[M - H + CH_3CN]^+$	+41
$[M + K]^+$	+38	$[M + HCOO]^-$	+46
$[M + CH_3CN + H]^+$	+41	$[M + CH_3COO]^-$	+60
$[M + H_2O + CH_3OH + H]^+$	+50	$[M + Br]^-$	+80
$[M + CH_3CN + Na]^+$	+63	$[M + HSO_4]^-$	+98
$[2M + H]^+$	–	$[M + H_2PO_4]^-$	+98
$[2M + Na]^+$	–	$[M + CF_3COO]^-$	+114
$[2M + K]^+$	–	$[2M - H]^-$	–

Mass differences refer to the difference between $[M + H]^+$ (left) or $[M - H]^-$ (right), respectively, and the related gas phase adduct.

and amount of mobile phase, the signal intensity of different gas phase species can change with the UHPLC flow rate.

In-Source Collision-Induced Dissociation Gas phase fragmentation reactions are a fundamental element of mass spectrometric experiments. Imagine an analyte that is excited to vibrations in a well-controlled manner: this molecule will then selectively break into pieces at the weakest bonds, thus creating a characteristic fragment spectrum. As long as this fragmentation reaction can be stimulated reproducibly, these fragmentation patterns provide you with a molecular fingerprint, which tremendously helps to identify a chemical compound. And even if you cannot afford to run your entire LC/MS experiment in full scan mode with parallel MS/MS fragmentation because you need highest sensitivity, the detection of as many known characteristic fragments as possible in SRM mode increases the level of confidence for your compound confirmation substantially. However, ESI and APCI are rather mild ionization principles transferring the analyte molecules into the gas phase as a whole, so fully intact and nonshattered – which is very different from EI (also earlier known as *electron impact ionization*) in GC/MS. The benefit is that you will be able to determine the molecular mass of the intact molecule, but you lose the chance to learn more about the structural properties and chemical nature of your analyte by fragmentation patterns. Sophisticated tandem-MS techniques, however, enable you to stimulate well-controlled decomposition conditions in the collision cell of a tandem mass spectrometer. The user typically knows about the most characteristic fragments of the target analyte, so not too many surprises are to be expected then. Many years of extensive research meanwhile allowed unveiling a huge set of decomposition reactions and their follow-ups in the gas phase; a very informative and comprehensive tutorial by Holcapek *et al.* is a valuable starting point for your own interpretation of small molecule fragmentations in API mass spectrometers [90].

Next to these intended fragmentation reactions in a tandem MS, the user will always have the chance – or the risk – to shatter the analyte in an uncontrolled way, typically stimulated by unfavorable ion source or ion transfer conditions. As long as the analytes have not entered the final high vacuum section of the mass spectrometer, that is, while they still are in the ion source or the transfer section of the ion optics that come along with a staged pressure reduction, these ions will have to remain intact in an environment where their mean free path is only in the range of a few micrometers (~50 µm at 1 mbar of ambient pressure) and not several dozens of inches (~20″/500 mm for a vacuum of 10^{-4} mbar). A collision with excessive ambient gas molecules is very likely there, and the higher the collision impulse, the more you will see an unwanted fragmentation taking place already in the entrance area of the ion source. You can master this process to some extent by a smart selection of acceleration voltages in your ion optics. High voltages, to be applied, for example, along the ion transfer capillary or to the skimmer electrodes, strongly accelerate the ions while traveling through the ion optics and induce more effective collisions with residual gas molecules, thus resulting in more fragment signals in the mass spectrum (also called *nozzle-skimmer dissociation*). In case

of the MS method development, it is a useful approach to change the parameter settings for the ion source and the transfer section stepwise while monitoring the signal change and the spectrum quality accordingly to avoid excessive analyte fragmentation. However, you can also make use of this principle to artificially stimulate ion fragmentation and to learn more about unknown compounds even with rather simple and cost-effective instruments such as single-quad mass spectrometers.

A very comprehensive table on the generation of fragments out of various functional groups can be found in the literature [90]. We briefly discuss the behavior of alcohols, aldehydes, and carbonic acids as representative examples for frequently occurring fragmentation reactions. These compound classes have a strong heteropolarity of the carbon–oxygen bond in common due to the high electronegativity of the oxygen atom. One immediate result of this is the neutral loss of water; the loss of carbon oxides is another one, depending on the chemical composition of the analyte. Once protonated in positive mode, alcohols preferably split off water ($R\text{-}OH_2^+$ from R-OH), thus generating an R^+ fragment (equaling $[M + H{-}H_2O]^+$), which is lighter than the expected ion of the intact molecular ion by nominal 18 Da. Aldehydes lose carbon oxide (CO), ending up in a fragment ion $[M + H{-}CO]^+$. Aliphatic carbonic acids typically lose the thermodynamically very stable carbon dioxide after protonation, while aromatic carbonic acids under the same conditions preferably "only" split off water, leaving us with an acylium cation for detection.

Contaminants Eluting from the Instrumentation Nevertheless, there will still be many situations where a good knowledge of gas phase fragmentation reactions and chemical expertise will not help to explain the existence of prominent mass signals in your MS spectrum: for instance, the sheer amount of mass signals being so high that they cannot be deduced by fragmentation reactions, or mass interferences not only showing up as one individual signal but as a series with distinctive patterns. The root cause here can be very trivial – contaminants are eluting from your chromatography or the mass spectrometer hardware. If your MS was thoroughly cleaned recently, then the (U)HPLC system and any fluidics connected with it would be blamed for that, and the potential contamination sources are numerous:

- *Bleeding* of a separation column being either old or unsuitable for MS detection results in an increased elution of the stationary phase bonding, which is split off the carrier material surface and leads to an increased noise in the LC/MS chromatogram.
- *Plasticizers* are ubiquitous in almost all modern plastic materials, from sample vials over solvent lines, piston seals to filter frits and many other pieces. Very common plasticizers are phthalates which can rapidly be identified based on their characteristic masses (m/z 279, 391, 413, 429, 454, and many more). Also lubricants and separating agents such as Erucamide (m/z 338, 360) can frequently be observed.

- *Polyethers* such as *polyethylene glycol* (PEG) and *polypropylene glycols* (PPG) are another frequent contamination type, which can be identified quickly due to their characteristic MS spectrum pattern. Also they are nearly ubiquitous in plastic materials, but they can also be introduced into the MS by working sloppily with disposable gloves or skin care products. These compounds never show up with only one single mass signal but always come with a very characteristic polymer distribution pattern [91]. The mass distance of the monomeric units are $\Delta m/z = 44$ for PEGs and 58 for PEGs, which immediately reveals the chemical nature of these contaminants.
- *Polysiloxanes (silicones)* are core ingredients of many modern high-performance oils and vacuum grease. Oil vapor traces from the rough vacuum pump leaking into the mass spectrometer produce characteristic signals of, for example, m/z 371, 445, or 519. In such a case, the ion optics, such as focusing multipoles or ion funnels, should be checked for cleanliness. A continuous stream of oil mist entering the MS can even lead to a razor-thin oil film coating the metal surfaces of the ion guides or, in some cases, on the mass analyzer over weeks and months. This results not only in contaminant signals in your mass spectrum but also in a measurable sensitivity loss, which makes a thorough and extensive cleaning mandatory in the end. But how would it happen anyway that oil mist from the rough pump(s) could enter the MS interior in a way that also comes along with ion generation? Well, the most obvious reason for this is a nonideal installation of the various exhaust hoses of your MS. For convenience, the exhaust tubes of the vacuum pump(s) and of the ion source drainage are frequently tied together into the same lab exhaust ventilation nozzle. But by doing so, you allow the vacuum pump exhaust to diffuse backward into the MS ion source and further down into the mass spectrometer. Installing the pump exhaust and the ion source draining tube into different connectors of the lab ventilation with a distance of 1.5–3′ (0.5–1 m) in-between is a very simple and effective solution to that issue.
- *Metal ions* can be a great origin for the generation of larger gas phase adducts with your target analyte molecules; alternatively, metal ions can also react with parts of your sample and thus inhibit the detection of compounds. We already discussed the formation of alkali metal adducts in section "Gas Phase Adducts"; A SST LC fluidics or massive hardware defects in a biocompatible UHPLC system, for example, a damaged injection valve, can result in a propagated release of iron ions and thus in iron/analyte clusters in the mass spectrum; these clusters reveal themselves very quickly due to their multicharge state and the isotope pattern of iron, which significantly deviates from those of the usual elements in organic matter such as carbon, hydrogen, nitrogen, and oxygen. Biochemical applications are particularly prone to issues created by heavy metals in the mobile phase. A lot of biological compounds tend to form either precipitates or nonvolatile aggregates with iron, or alternatively to irreversibly adsorb on iron surfaces. A frequently described phenomenon is the "vanishing," so the nondetectability of phosphorylated peptides and

proteins in a separation system with an SST fluidics. To avoid this, bioanalytical applications are preferably run on instrumentation with an iron-free fluidics, which can be made of titanium, biocompatible metal alloys like MP35N, or PEEK, with the latter being not very pressure-resistant (tubing typically up to 5–6000 psi/350–400 bar) and thus not being suitable for UHPLC applications.
- *Dissolved residual gases* in the mobile phase, however, typically result in an unstable spray and/or interfering spikes in the LC/MS chromatogram and the mass spectra rather than modifying the analyte mass signals.

The aspects discussed here can only represent a selection of some very popular phenomena, but this list is far away from being a fully comprehensive compilation of known interference signals [88]. Already some years ago, Keller *et al.* published an excellent and highly detailed, tabulated collection of all literature-known MS contaminants known at this point [92]. Most MS vendors discuss this topic very openly as well and compiled various collaterals on MS contamination sources [89, 93], and last but not least the internet offers various public data search engines on that matter. One example to be mentioned is the *MaConDa* (*Ma*ss spectrometry *Con*taminant *Da*tabase) database maintained by the University of Birmingham [91], which allows to search for more than 300 contaminants based on accurate mass, compound class, and mass spectrometer type (operation principle and manufacturer), featuring also many literature references.

1.2.3.5 Instrumental Reasons for the Misinterpretation of Mass Spectra

Finally, we investigate some selected instrumental reasons that can lead to the misinterpretation of mass spectra. As discussed earlier, all MS types have their strengths and weaknesses that affect also the quality of your analytical result. Let us discuss these in the following on three scenarios:

False Mass Assignment Depending on Ionization Principle As already discussed in section "Gas Phase Adducts", the selected ion creation principle determines which m/z value is shown in your mass spectrum for an unknown analyte species. The most frequently used ionization process by charge transfer, that is, proton association or distraction (used in ESI and APCI), does not result in the molecular mass of the intact molecule being measured, but in an m/z value differing by one proton mass (or multiple proton masses at a respectively corrected fraction of the intact molecule mass for multicharged molecular ions). Other processes such as APPI or EI (hardly used in LC/MS), which can create ions also by transferring electrons instead of protons lead to a measured m/z value, which deviates from the theoretical value of the intact molecule only by the much smaller mass amount of an electron. With ESI, APCI, and APPI, the formation of adducts with alkali metals and/or residual solvent molecules can lead to misinterpreting a numerical m/z value as representing an $[M+H]^+$ ion species, which in fact would be, for instance, an $[M+H_2O+Na]^+$ ion instead. A thorough look on your mass spectrum can be very helpful here, as many

adduct species coexist with others, with characteristic m/z differences between the various ion aggregates. So if you find a new m/z value, which you may take for an $[M+H]^+$ ion, just check for further signals with m/z differences to the first one of, say, $[\text{“M+H”}+22]^+$, $[\text{“M+H”}+38]^+$, or $[\text{“M+H”}+41]^+$ – with these being a sodium, potassium, and ACN/proton adduct, respectively; this series can additionally confirm (or disprove) your originally assumed mass assignment.

False Interpretation Due to Poor Mass Resolving Power Another reason leading to a mass signal misinterpretation is a poor or inappropriate mass resolution. Imagine two different analyte species having only very small differences in their m/z values and arriving in the mass analyzer simultaneously; a low-resolving mass spectrometer will not be able to sufficiently discriminate the two different masses. The resulting mass spectrum will thus show only the envelope curve for the two different mass patterns, and the peak maxima of this envelope function do not necessarily have to be identical to the mass signal maxima of the mass spectrum of each individual compound. Also, low-resolution mass spectrometers will not be able to resolve higher charge states; we know that the m/z distance between the isotope pattern signals of a compound equals the $1/n$th fraction of the charge state n, which means at the same time that for analyte ions with three or more charges the isotope pattern cannot be resolved appropriately with low-res mass specs. In summary, co-eluting contaminants or impurities could not be identified as such, or mass signals are erroneously assigned to the wrong compounds. It depends – next to your budget of course – on the sample complexity and the quality of your separation how good the resolving power of your mass spectrometer must be at minimum. The better your chromatography, the more unambiguous the interpretation of your mass spectrum will be in the end. As a general recommendation, a mass spectrometer for the determination of accurate masses should have a resolving power R of 10 000 – 15 000 at least; the scientific literature typically defines R of 10 000 as the minimum for high-resolution and R of 100 000 for ultra-high-resolution mass spectrometry [75].

False Mass Determination Due to Inappropriate or Unstable Experimental Conditions It is an obvious fact: a mass determination is always achieved by comparing your instrumental MS data for the analyte with the mass signals of known calibration standards; thus the quality and the long-term stability of the mass calibration are critical for a reliable mass measurement. Expired or contaminated reference standards with partially degraded ingredients should of course not be used for calibration anymore. An undefined number of mass signals during the mass calibration process compromises the correct mass assignment and impairs or even blocks software tools such as autotune algorithms. Once successfully calibrated, the quality of the mass axis calibration needs to be verified regularly. As discussed earlier, TOF instruments are particularly prone to drifts in the mass axis calibration even on short-term periods of (much) less than 1 h. Hence, an internal mass calibration by a continuous calibrant infusion is essential for a

reliable mass determination. Mass spectrometers of the ion trap design (QIT, LIT, FTICR, Orbitrap) are additionally influenced by the spatial density of the ions stored in the electromagnetic field cage of the ion trap. The circulating ion packages in the trap act as so-called *space charges*, which induce additional electrical fields, shielding and thus locally distorting the external electromagnetic trapping field. Higher charge densities, which means high amount of analyte ions in the trap cell and/or multiply-charged ion species, translate then into a pronounced shift of the resonance conditions for your circulating ion packages, which impairs mass accuracy and mass resolution significantly. An overfilled ion trap will then give you shifted and thus falsified mass signals. In contrast, the fewer ions you trap, the more you will lose sensitivity. The ideal filling degree for an ion trap is dynamically calculated in real time by modern MS control software – feature names here are *ICC*, *AGC*, or others, depending on the MS vendor. Very concentrated samples, however, cannot always fully be intercepted by the control algorithms and thus will still lead to a short-time overfilling of the ion trap. Due to their design principle with a stretched longitudinal cell construction, LIT typically suffer less from space charge effects than circular traps (QIT).

1.2.4 Conclusion

For almost two decades, the coupling of LC and mass spectrometry (MS) has been successfully commercialized now. With the first instrument generation being true divas requiring in-depth expert knowledge, nowadays this technology has reached a fairly mature development state, which substantially lowered the entry barrier to this technique; this results in many robust LC/MS solutions being established in the market, and LC/MS is more and more penetrating the field of routine applications, as users do not have to adopt a high amount of expertise to create quick and reliable results. Within these 20 years of growth and evolution, not only new MS technologies such as the Orbitrap have seen the light of day but also LC has made a big step ahead by moving from HPLC to UHPLC with much higher separation efficiencies and shorter run times. So from a bird's perspective, LC/MS has evolved to a very powerful analytical tool, which is surprisingly easy to use given the high complexity of the technologies involved. But nevertheless, mass spectrometry is not the analytical all-purpose weapon as it is advertised in some cases, and it will not be for a long time. Key to the highest analytical benefit of an LC/MS installation is the thorough mutual physicochemical optimization of the LC and the MS world; this chapter hopefully is one contribution to better understand the technical needs and to avoid the most general pitfalls. As the scientific progress is moving forward, several new technological territories will be entered in the future for sure, be it for even higher speed and resolution, for instrument miniaturization, or for enhanced usability by new and powerful software tools. However, one thing is for sure: it is not only the liquid phase separation that

benefits from the mighty analytical information creation by mass spectrometry but also MS technologies will massively fall short on their potential without a thoroughly optimized chromatography upfront. For the foreseeable future, both concepts, chromatography and mass spectrometry, will continue to depend on each other.

1.2.5 Abbreviations

AP(C)I	atmospheric pressure (chemical) ionization
CAD	charged aerosol detection
CID	collision-induced dissociation
ECD	electrochemical detection
EI	electron ionization (also: electron impact ionization [obs.])
EIC	extracted ion chromatogram
ESI	electrospray ionization
ELSD	evaporative light scattering detector
FA	formic acid
FIA	flow injection analysis
FT	Fourier transformation
GDV	gradient delay volume
HPG	high-pressure gradient pump
HR/AM	high-resolution/accurate mass
HTS	high-throughput screening
HV	high voltage
ICR	ion cyclotron resonance
I.D.	inner diameter
LIT	linear ion trap
LPG	low-pressure gradient pump
MRM	Multiple Reaction Monitoring
MSD	mass selective detector
PEEK	poly(ether ether ketone)
PEG	poly(ethylene glycol)
PPG	poly(propylene glycol)
QIT	quadrupole ion trap
SIM	single-ion monitoring
SRM	Selected Reaction Monitoring
SST	stainless steel
TFA	trifluoroacetic acid
TOF	time of flight

1.3
LC Coupled to MS – A User Report

Alban Muller and Andreas Hofmann

In comparison to coupling LC to UV, coupling LC to mass spectrometry (MS) requires important adaptations. Water–methanol or water–acetonitrile gradients are often applied in RP chromatography. Only volatile buffers should be used in order to prevent contamination of the MS instrument by nonvolatile salts. For example, FA or acetic acid can be used for acidic conditions and ammonia can be used for alkaline conditions. If samples contain nonvolatile salts, the LC flow can be directed to waste at the beginning of the analysis to prevent contamination of the MS instrument. A two-dimensional LC system with an enrichment column is another option to analyze samples containing nonvolatile salts. Matrix effects can reduce signal-to-noise ratios of analytes in complex biological samples. Adding heavy isotope-labeled standards to samples allows to take losses during extraction and matrix effects into account. ^{2}H, ^{13}C, and ^{15}N are often used to isotopically label standards. In contrast to ^{2}H-labeled standards that can show small retention time shifts, ^{13}C- and ^{15}N-labeled standards elute at the same time as the analytes.

The most suitable LC system can be selected based on the required sensitivity and the desired analysis time. The MS signal intensity is proportional to the concentration of the analyte. Nano LC systems are mainly used to achieve highest sensitivity. High sensitivity is especially critical for the analysis of low abundant endogenous molecules, for example, in proteomics experiments. In proteomics experiments, chromatographic columns have often an inner diameter of only 75 µm or lower. The low solvent flow rate (e.g., 250 nl/min) in nano-LC/MS methods can lead to an analysis time of 1 h and more, but allows the most sensitive approach to detect endogenous molecules. Higher LC flow rates (400–600 µl/min) are usually applied for the detection and quantification of analytes with higher concentrations. Chromatography columns with a 1 or 2.1 mm inner diameter are often used for U(H)PLC applications that allow to complete an analysis in only few minutes.

The method of ionization is chosen based on analyte properties and the LC flow rate. ESI is suitable for a very broad spectrum of analytes and LC flow rates. Hydrophilic as well as hydrophobic substances can be well analyzed with ESI. APCI and APPI are mainly applied for the analysis of very hydrophobic substances and require high LC flow rates. In general, the ion source temperature and the gas flow rates need to be increased with increasing solvent flow rates. Recommended parameters for different LC flow rates can be found in the manual of the ion source. These recommended parameters often represent a good starting point for your own optimization. In addition to parameters that depend on the solvent flow rate, analyte-dependent parameters of the ion source need to be optimized. Analyte-dependent parameters can be optimized by direct infusion of a pure analyte solution with a syringe pump, in order to adjust all parameters for a maximal signal-to-noise ratio. Combining the flow of the syringe pump via a T-piece with the LC flow allows to simulate conditions close to the final

analysis conditions. Relatively high concentrations of analyte have to be used for direct infusion, which can lead to background signals of the analyte. Different instrument parameters can also directly be varied during the LC/MS analysis, due to the short analysis time of U(H)PLC systems and the fast scan speed of modern MS instruments. A lower concentration of analyte can be used for the variation of parameters during the LC/MS analysis compared with direct infusion. Many steps of the optimization can automatically be done by the instrument software or platform-independent software. However, automatically determined parameters should always be checked for plausibility. Orifices of MS instruments have been enlarged over the last years in order to increase ion transmission. Not only more ions but also more neutral particles enter the MS instrument through the enlarged orifice; therefore, strong roughing and turbo pumps are necessary to maintain the required vacuum. Furthermore, the geometry of the ion optics were changed to efficiently separate ions from neutral particles, for example, by using the StepWave™, iFunnel, or electrodynamic ion funnel technology. The improved ion transmission of modern MS instruments results in higher signal-to-noise ratios, more robust methods, and facilitated optimization of instrument parameters.

Mass accuracy, mass resolution, scan speed, sensitivity, and many other parameters of MS instruments can differ greatly. Some applications may only be feasible with one specific type of MS instrument. Basic MS instruments, such as single quadrupole, ion trap, or TOF instruments, can be used to analyze samples with low complexity. Hybrid MS instruments, combining two mass analyzers, are often used to analyze complex biological samples. Hybrid MS instruments with high mass accuracy and mass resolution, for example, Q-TOF, TOF-TOF, ion trap-Orbitrap, or Q-Orbitrap instruments, allow the identification of unknown substances. Triple quadrupole MS instruments with high scan speed and excellent sensitivity are often applied for quantitative analyses. A triple quadrupole instrument consists of three quadrupoles arranged one after the other (Figure 1.12a). The first quadrupole is used as a mass filter for the ionized, intact analyte. The ionized, intact analyte is also called parent ion. The parent ion is then fragmented in the second quadrupole by collision-induced dissociation. Subsequently, the third quadrupole filters for a specific fragment of the parent ion, also called daughter ion. Assays on triple quadrupole instruments are very sensitive and selective due to the double filtering in the first and third quadrupole.

For example, isomers of the hydroxy-eicosatetraenoic acid are difficult to separate chromatographically (Figure 1.12b), and a simple mass analysis shows overlapping peaks for the parent ions (Figure 1.12c). However, a triple quadrupole instrument enables to measure specific daughter ions of the two isomers and the daughter ion traces show no interferences between the two isomers [94].

Ion-pairing reagents, such as TFA for acidic conditions or triethylamine for alkaline conditions, can be used for the separation of hydrophilic analytes by RP chromatography. In general, ion-pairing reagents often lead to a reduced signal-to-noise ratio and to high background signals when the polarity is switched.

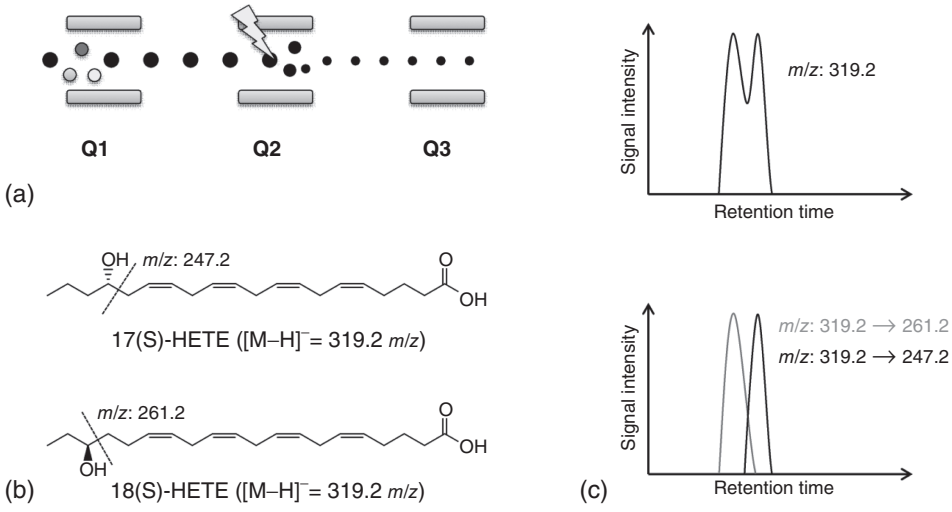


Figure 1.12 (a) Schematic representation of the quadrupoles of a triple quadrupole instrument. Only two quadrupole rods of the four quadrupole rods are shown for each quadrupole. The first quadrupole (Q1) filters for the precursor ion, the precursor ion is fragmented in Q2 by collision-induced dissociation and Q3 filters for a specific daughter ion. (b) Isomers of hydroxy-eicosatetraenoic acid (HETE). (c) Schematic representation of ion traces by measuring just the precursor ions or daughter ions of HETE isomers.

Ion chromatography (IC) is a reliable alternative to separate very hydrophilic, charged molecules, which cannot be readily analyzed by RP chromatography. Analytes are separated based on their charge and size by IC. In contrast to silica-based RP columns, the stationary phases of IC columns are polymer-based. Therefore, IC columns are very stable under alkaline conditions. Potassium hydroxide is often used for anion exchange chromatography, and the eluting strength is directly proportional to the potassium hydroxide concentration. Methanesulfonic acid is often used for cation exchange chromatography.

An example of an anion exchange IC coupled to a triple quadrupole MS is described in the following section. A potassium hydroxide gradient is produced by the eluent generator and analytes are separated on the IC column. Thereafter, potassium ions are exchanged by hydronium ions in the electrochemical suppressor. Usually, IC is coupled to a conductivity detector, which determines the conductivity of the solution. A conductivity detector is relatively insensitive and not well suited for biological applications, for example, in the field of metabolomics. Furthermore, the conductivity is not selective so that analytes with the same retention time cannot be distinguished. In contrast, a triple quadrupole instrument enables a very sensitive and selective detection of different analytes. Without electrochemical suppressor, potassium ions would lead to a strong suppression of analyte signals and contamination of the MS instrument. The electrochemical suppressor generates an aqueous solution with a low salt concentration of a few microsiemens. The efficiency to generate negative ions

can be increased by adding an organic solvent, such as methanol, via a T-piece to the aqueous solution. A Dionex ICS-3000 IC system, an ESI ion source and an AB Sciex QTrap 5500 MS instrument was used for the analysis described here. The triple quadrupole instrument allows a very sensitive and selective detection of analytes via MS/MS experiments.

1.3.1 Conditions of the Ion Chromatography

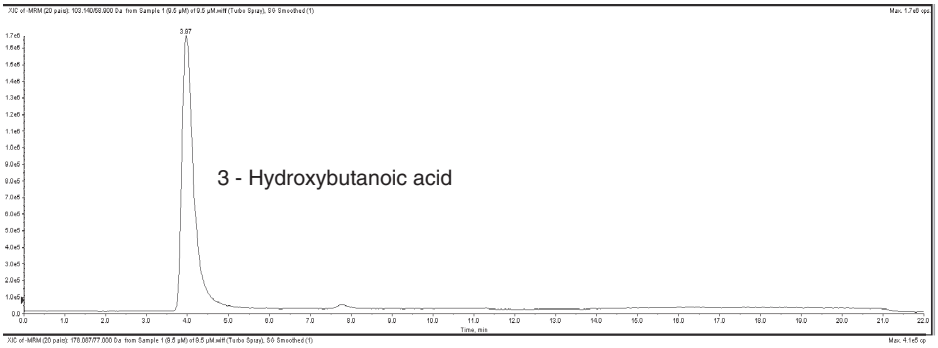
Eluent generator	EGC III KOH
Enrichment column	Ion Pac AG20 2 × 50 mm
Analytical column	Ion Pac AS20 2 × 250 mm
Column temperature	35 °C
IC pump	Isocratic, 250 µl/min
Suppressor electric current	62 mA
Loop volume	2 µl
Methanol flow rate	50 µl/min

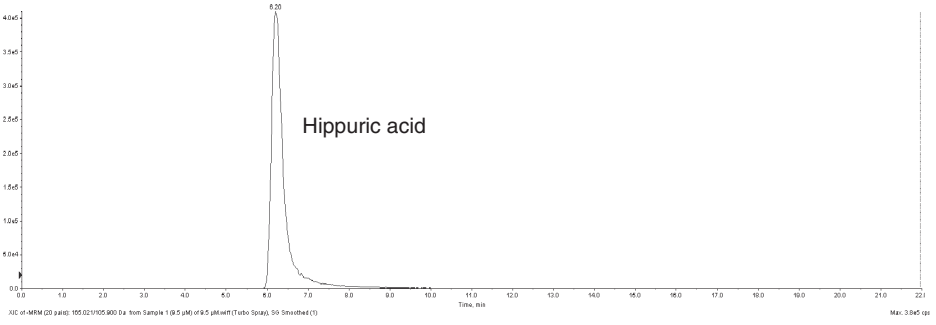
1.3.2 Gradient Generator

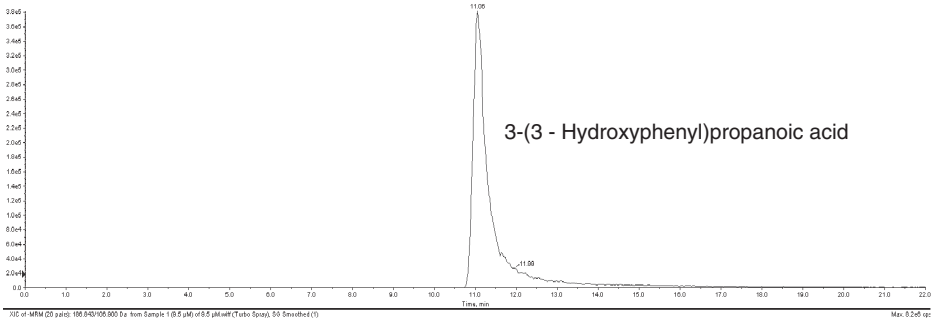
Time (min)	$[OH^-]$ (mM)
0	10
7.5	45
17.5	48
18.5	100
22.5	100
22.6	10
25	10

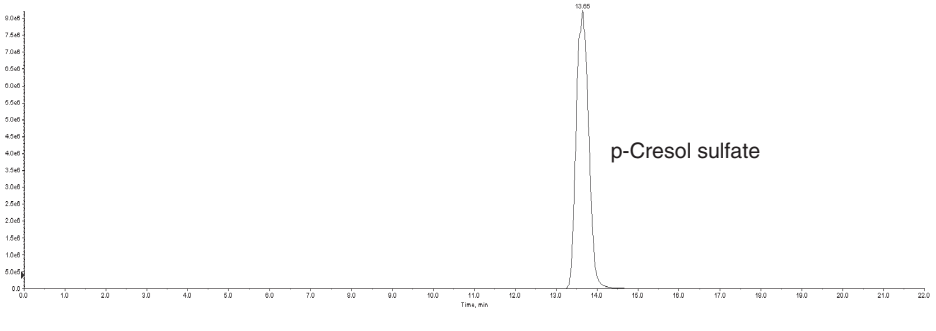
1.3.3 Transitions

Analyte	Q1 mass (Da)	Q3 mass (Da)
3-Hydroxybutanoic acid	103.1	58.9
Hippuric acid	178.1	134.1
3-(3-Hydroxyphenyl)propanoic acid	165.0	105.9
p-Cresol sulfate	186.8	106.9


3.97
3 - Hydroxybutanoic acid
Time, min


6.20
Hippuric acid
Time, min


11.05
11.58
3-(3 - Hydroxyphenyl)propanoic acid
Time, min


13.65
p-Cresol sulfate
Time, min

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