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Current Developments in LC-MS for Pharmaceutical Analysis

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Abstract

Liquid chromatography (LC) based techniques in combination with mass spectrometry (MS) detection have had a large impact on the development of new pharmaceuticals in the past decades. Continuous improvements in mass spectrometry and interface technologies, combined with advanced liquid chromatographic techniques for high-throughput qualitative and quantitative analysis, have resulted in a wider scope of applications in the pharmaceutical field. LC-MS tools are increasingly used to analyze pharmaceuticals across a variety of stages in their discovery and development. These stages include drug discovery, product characterization, metabolism studies (*in vitro* and *in vivo*) and the identification of impurities and degradation products. The increase in LC-MS applications has been enormous, with retention times and molecular weights (and related fragmentation patterns) emerging as crucial analytical features in the drug development process. The goal of this review is to give an overview of the main developments in LC-MS based techniques for the analysis of small pharmaceutical molecules in the last decade and give a perspective on future trends in LC-MS in the pharmaceutical field.

Keywords

Liquid chromatography-mass spectrometry (LC-MS); pharmaceutical analysis; small pharmaceutical molecules; interface technologies; separation techniques

1. Introduction

The process of developing a new drug from first concept to the launch of a commercial product, is long and complex, and can easily take more than a decade and cost over 1 billion Euro. During this process, an intensive search for a drug-like small molecule or biological therapeutic (target) takes place. Once a target has been chosen, the pharmaceutical industry, often with the help of academic centers, uses streamlined early processes to identify compounds with suitable characteristics to make acceptable drugs[1]. Genetic, cellular, and *in vivo* tools using animal models are part of the drug discovery preclinical screening process to validate a drug product before testing it as a candidate molecule for clinical development.

The safety of a drug product depends on the toxicological properties of the active substance, and its impurities and degradation products. The quality and quantity of the drug product and the substances used during its manufacturing are typically determined via different analytical methods, such as titrimetric, chromatographic, spectroscopic, and electrochemical methods[2]. Among the chromatographic techniques, liquid chromatography (LC) plays an important role in pharmaceutical industries and analysis. Since its first application in 1980 for the assay of bulk drug materials, LC has become one of the principal methods in both the United States and European Pharmacopoeia[3]. Due to their high resolving power, LC-based techniques help in the structure determination and quantitation of degradation products and impurities in bulk drug materials and pharmaceutical formulations. In this respect, the choice of the detector is critical to guarantee that all components can be detected. The ultraviolet (UV) detector is still the most commonly used detector in LC. It is capable of monitoring several wavelengths simultaneously by applying a multiple wavelength scanning program. However, it can rarely provide an unambiguous identification.

The addition of further detection capabilities to LC separations offers unique opportunities to support pharmaceutical development efficiently and ensure the quality and safety of pharmaceuticals[4]. The hyphenation of LC and mass spectrometry (MS) has become very important in drug discovery and development due to its high throughput, selectivity and sensitivity[5–7]. LC-MS combines the separation power of LC (production of pure or nearly pure fractions in a chemical mixture) with the mass analysis capabilities of MS (identification of compounds with high molecular specificity and detection sensitivity). This orthogonal hyphenation provides unique solutions to a wide range of structural characterization problems, resulting in the most powerful analytical tool for non-volatile compounds today[8]. Continuous improvements in LC-MS interface technologies, together with powerful tools for structural analysis have led to a wide range of applications in all stages of drug development (discovery, preclinical and clinical phases), metabolism studies (*in vitro* and *in vivo*), and for the identification of impurities and degradation products[9].

The aim of this contribution is to give an overview of the main developments in LC-MS for the analysis of small pharmaceutical molecules in the past decade (2010-2019). Among the most important trends, ultra-high performance (UHP) LC, multidimensional (MD) LC, high-throughput LC-MS, as well as various technical aspects, such as miniaturization and microfluidics, new MS interfaces, and their coupling with LC systems are critically examined in the context of pharmaceutical analysis. Strategies to minimize matrix effects and improve sensitivity are briefly discussed. Finally, a short summary of recent applications of LC-MS in the field, such as drug metabolism, drug discovery, chiral impurities, degradation products and pharmacokinetic/pharmacodynamic (PK/PD) studies, is also reported.

2. Recent Developments in LC-MS

2.1. Ionization

The type and the design of the ionization source has an important impact on the performance of an LC-MS method. Electrospray ionization (ESI) is still the preferred ionization method in pharmaceutical analysis for the analysis of thermally labile, nonvolatile and polar compounds[5–7,10], followed by atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI) for nonpolar or less polar compounds with lower molecular weights[11–13]. Since most small molecule drugs are weak bases and hence easily protonated, pharmaceutical analyses are often performed in positive ionization mode. For acidic functional groups that easily loose a proton, negative ionization mode can be applied. Several instruments can nowadays also switch polarity within several tens of milliseconds, making the simultaneous analysis of compounds ionized in different ionization polarity, possible[14–16].

Due to the complementarity of ESI and APCI, the use of multimode ionization sources has been proposed for the LC-MS analysis of samples containing analytes with a wide range in polarity and volatility. Such multimode ionization sources allow the use of ESI and APCI, either individually or simultaneously, in a single analytical run without interruption, hence increasing the analytical throughput. As an example, a multimode ionization source was used in ESI(+), APCI(+) and simultaneous ESI-APCI(+) mode for the LC-MS analysis of nine benzodiazepines and zoldipem[17]. It was demonstrated that ESI was more sensitive than APCI resulting in S/N values that were on average 1.7 times higher, while the simultaneous ESI-APCI ionization source was the most sensitive, resulting in S/N values that were on average 2 times higher compared to APCI. These findings suggested a possible additive effect between ions generated by ESI and APCI.

Although APPI is an attractive alternative to ESI and APCI due to its applicability for a broad range of compounds, it is currently not widely used as a result of its limited sensitivity. To enhance the ionization efficiency of the analytes, and improve the sensitivity of the analysis, a dopant is often added to the mobile phase. This, however, makes APPI more complex to use and less generic. In contrast to ESI and APCI, APPI can also be applied in combination with normal-phase conditions wherein flammable mobile-phase solvents are used, since there is no explosion hazard as can be the case with APCI or ESI. In this respect, it has been demonstrated that some of these mobile phase solvents (e.g., hexane-based mobile phases) are self-doping and hence make the addition of dopants redundant[18]. In modern commercial APPI sources, ionization and important secondary ion-molecule reactions occur in an open source environment close to the MS sampling orifice. It has been suggested by McCullogh et al. that this open-geometry configuration can strongly influence the ion-molecule chemistry that is crucial for APPI performance, and the efficiency with which ions are transmitted from the source to the MS interface[19]. Therefore, the authors suggested to revisit the field-free closed-geometry design that was developed at the same time as the open-geometry configuration. In the closed-geometry design, ion-molecule reactions occur within a closed stainless-steel source block, leading to the formation of analyte ions. When these ions exit the source block, they are transmitted to the MS sampling orifice through a strong electric field that is established between the source block and the MS interface. While transiting through the source block, there is no electric field present (“field-free”), which allows reagent ions and analyte neutrals to travel together for an extended duration[19]. The field-free APPI source (FF-APPI) was compared with commercially available open-geometry source designs and yielded a significant sensitivity advantage (5- to 10-fold increase) for the analysis of two neutral steroids. This enhanced sensitivity was attributed to the improved confinement of the ion beam and the increased ion-molecule reaction time in the field-free source. Detection limits obtained with FF-APPI for steroids such as estrone, estradiol and androsterone, were compared to ESI and APCI and were up to 10x lower. Finally, matrix effects were evaluated for complex biological samples, such as human serum, plasma and simulated urine. Matrix effects (ion suppression) in FF-APPI seemed to be enhanced at elevated vaporizer temperatures and at low nebulizer gas pressure, although analyte

sensitivity was improved at reduced nebulizer pressures. Further experiments demonstrated that ion suppression could be almost completely eliminated at high nebulizer pressures (80 psi), however, at the cost of a decreased analyte signal intensity[20].

As an alternative to ESI, APCI and APPI, a new atmospheric pressure ionization source, called UniSpray, was recently introduced by Waters Corporation. The UniSpray source consists of a grounded nebulizer spraying onto a high-voltage, stainless steel rod located between the sprayer and the ion inlet orifice of the mass spectrometer. The ionization mechanism of the UniSpray source is similar to ESI, but benefits from additional Coanda and vortex effects to promote droplet break-up and desolvation[21]. The sensitivity of the UniSpray source can be optimized by adjusting the point at which the spray impacts the stainless-steel rod. Maximum source sensitivity is typically obtained when the spray impacts the upper right quadrant of the target, since this leads to the occurrence of asymmetric gas streamlines that are directed towards the ion inlet orifice. The performance of the UniSpray source was compared to ESI on the same MS by direct infusion of a mixture of pharmaceuticals with a variety of physico-chemical properties and indicated similar ionization behavior for both sources, with the formation of the same adducts in similar ratios. When comparing the signal intensity obtained with UniSpray versus ESI for different solvent compositions (percentages of organic modifier and pH) and flow rates, a net gain of 2.2 was generally obtained with the UniSpray source[22]. When coupled with LC, the UniSpray source demonstrated a slightly improved dynamic range towards lower concentrations. Matrix effects (MEs) were also investigated and compared between UniSpray and ESI for polymer excipients, plasma, bile and urine and were found to be similar for both sources[23]. Ciclet and coworkers reported another comparison between UniSpray and ESI coupled with LC in both positive and negative ionization mode in terms of sensitivity, stability, versatility and MEs, using 120 natural compounds covering a broad chemical space. In terms of sensitivity, MS intensities of around 40 natural compounds improved using UniSpray versus ESI, but the benefits of UniSpray ionization strictly depended on the chemical class of the investigated analytes. For some compounds, a 10-fold increase in sensitivity was observed using UniSpray versus ESI, while 23% of the compounds had higher MS signal intensities using ESI. One third of the compounds showed equal sensitivities with ESI and UniSpray ionization[24].

Another recently introduced ionization method is Electrospray Ionization Inlet (ESII), wherein a voltage is applied to a metal union that connects tubing from a solvent delivery system such as an LC pump to a piece of fused silica tubing that is inserted into the heated inlet tube of a mass spectrometer. No nebulizing or desolvation gases are required, while the voltages necessary to obtain maximum sensitivity are typically less than 2.5 kV and hence lower than in ESI. The sensitivity of ESII has been compared to ESI for a number of small molecule drugs on an LTQ Velos mass spectrometer. In ESII, ion abundances of the base peak chromatograms were on average 3-10 times larger than those obtained from the extracted ion chromatograms with ESI for flow rates ranging between 15 and 50 µL/min[25]. In contrast with the Unispray source, the gain in ion abundance was characterized by lower background ions. It should further be noted that ESII performs better at low flow rates, while Unispray is mainly beneficial at higher flow rates.

All atmospheric pressure ionization techniques suffer from a number of inherent disadvantages such as non-uniform, compound specific responses, limited ionization of non-polar compounds and susceptibility to signal suppression or enhancement, depending on the sample matrix[26]. The latter phenomenon, also called matrix effects (MEs), can strongly impact the reproducibility, linearity and accuracy of the method, resulting in an unreliable quantitation. MEs in ESI can occur through different processes, such as the competition between matrix components and target analytes for the available charges in the liquid phase. When interfering compounds are present at high concentrations in the

matrix, they can also influence the viscosity and surface tension of the droplets that are formed in the ionization interface, hence influencing the transfer of target analytes to the gas phase. Alternatively, analytes can co-precipitate with non-volatile matrix components, which will reduce their capacity to reach the gas phase. Finally, once in the gas phase, the analyte can lose its charge due to neutralization reactions or charge transfer in the presence of interfering compounds[27].

In APCI, the analyte molecules are first transferred to the gas phase as a neutral molecule and ionization only occurs in a second step by chemical ionization. This makes that most mechanisms leading to ion suppression in ESI are absent in APCI, resulting in a lower susceptibility to matrix effects. Ion suppression in APCI can still occur, however, for example by the formation of solid precipitates by non-volatile compounds or by the effect of the sample composition on the efficiency of the charge transfer from the corona discharge needle to the analyte. In APPI, ion suppression is probably due to competitive gas-phase ion-molecule reactions. Matrix compounds can either capture the charge from the chemical reagent ion supply or charge can be removed from previously ionized analytes by directly interacting with basic or electrophilic matrix components.

To overcome MEs, the use of electron ionization (EI) MS in combination with LC has been proposed as an alternative to atmospheric pressure ionization techniques. Electron ionization operates via a different ionization mechanism, wherein liquid is completely converted to the gas phase prior to ionization through interaction with an electron beam under high-vacuum conditions. The steps leading to analyte ionization are influenced neither by matrix compounds nor the mobile phase composition. To induce analyte ionization, 70 electron volt (eV) electrons are typically used to transform the analyte into a radical cation. These radical cations undergo single or multiple fragmentations and rearrangements that are well-characterized and predictable. The *m/z* ratio and intensity of the parent and fragment ions can hence serve as a fingerprint that can be entered in a spectral library for identification against thousands of other library entries obtained under similar conditions. Since the identification potential of library searchable EI spectra, without the drawback of matrix effects, is very interesting for molecules in a liquid phase, several propositions have been made to ionize analytes from a liquid effluent using EI.

In Direct-EI, a low flow rate nano-LC system is coupled to a high vacuum EI source. Nebulization and vaporization of the liquid flow are carried out in the ion source under high-temperature, high-vacuum conditions. An EI ion source is typically made from stainless steel (SS). Adsorption and thermal degradation on the metal surface of the ion source can have a severe impact on the sensitivity and reproducibility of the detection signal, especially for polar, low-volatility and thermally sensitive compounds. It has been demonstrated that ceramic coating of the inner surface of the stainless steel ion source significantly improves the MS signal[28]. Both for the direct flow injection analysis of 16 polyaromatic hydrocarbons (PAHs) and the Direct-EI-LC-MS analysis of four hormones, peaks obtained with a ceramic coated ion source were much sharper and displayed significantly higher signal-to-noise (S/N) ratios (up to a four-fold increase) compared to a stainless-steel ion source. The application of the Direct-EI LC interface in combination with MS/MS was also demonstrated for the targeted detection and quantification of benzodiazepines in alcoholic drinks[29]. For this purpose, a fast direct-injection method requiring no prior sample preparation was proposed and validated, resulting in detection limits (LOQ) between 100 and 500 µg/L when injecting 2 µL of sample.

For the untargeted analysis of complex liquid matrices under routine conditions, the group of Cappiello developed another interfacing mechanism for LC-MS based on electron impact (EI), called liquid-EI or LEI[30]. In LEI, the LC eluate is vaporized inside a vaporization microchannel at atmospheric pressure before entering the ion source (Figure 1a). In this way, solute vaporization occurs near the ion source in an environment that is free of electric potentials. The microchannel, that can be heated to 400°C, is

obtained by lining a standard GC transfer line with a removable fused silica capillary – the liner – to prevent contact between the analytes and the metal surface. A narrower fused silica capillary – the inlet – protrudes the first part of the liner and releases the LC eluate. The eluate will evaporate when it comes into contact with the hot zone of the microchannel. Gas-phase ions that are formed will then move towards the ion source following the pressure gradient between the end of the LC capillary and the high-vacuum zone of the ion source. A helium flow is additionally passed between the inlet capillary and the liner and carries the gas-phase molecules to the ion source. Due to the small size of the microchannel, the residence time of the analytes in the hot zone is minimized, reducing the risk of thermal degradation. This increases the range of applications, especially with respect to nontargeted analyses. LEI ideally works at flow rates between 300 and 800 nL/min and is hence designed to be compatible with nano-LC separations. In order to make the ionization interface compatible with normal bore LC, a flow split needs to be applied post-column.

The proof-of-principle of the LEI interface was demonstrated for a mixture of 20 environmental priority pollutants with a wide variety in polarity (including caffeine and an estrogen). These compounds can currently not be analyzed at once using commercially available LC-MS equipment. Comparison of the average mass spectra of each compound with an electronic mass spectral library demonstrated that the mass spectrometer, equipped with the LEI interface, was able to generate EI mass spectra with an excellent quality. The applicability of LEI for untargeted analysis was demonstrated by analyzing a brain sample from a victim of sudden infant death syndrome and revealed the presence of benzo[a]pyrene as an environmental toxic contaminant. Recently, the same research group reported some changes in the proposed LEI interface including a modified geometry of the vaporization area. These modifications allow a better control of the heat distribution and an efficient liquid-to-gas conversion[31].

A disadvantage of Direct EI and LEI is that LC column flow rates are restricted to < 1 µL/min. This requires the use of nano LC columns, resulting in a lower sample loading capacity and loss in sensitivity. Direct EI-LC-MS and LEI moreover use 70 eV EI which is not always ideal since not all library compounds have a molecular ion[32] and LC-MS compounds cannot always be produced with a molecular ion of sufficient abundance, making a correct sample identification ambiguous. In their search for an ionization method that provides library searchable EI fragments in combination with an enhanced molecular ion, Amirav et al. have improved their supersonic molecular beam (SMB) approach for the combination of LC-MS and EI[33]. This was done to tackle the poor robustness that was observed for their original system and to extend the useful solvent flow rate range beyond 50 µL/min.

In the optimized EI-LC-MS with SMB system, the solvent enters via a Z-axis capillary tune device to position the solvent delivery capillary in the spray nozzle (Figure 1b). A Z-axis spray probe tune device allows to adjust the position of the spray nozzle with respect to the entrance of the heated vaporization chamber. A deactivated glass liner that is separately heated and controlled, functions as the heated vaporization chamber. The vaporization chamber is air-cooled at its entrance to prevent the liquid eluens from the LC of forming premature air bubbles that can result in undesirable spray instability. The liquid is pneumatically converted into a spray and the sprayed solvent and sample compounds are fully vaporized. Helium gas is used for nebulization and as a sheath gas, and sweeps the vaporized sample into a separately heated and temperature controlled deactivated fused silica capillary transfer line that separates the high-pressure vaporization chamber and the nozzle. The supersonic nozzle expands the vaporized solvent and sample compounds into a vacuum chamber together with helium gas and make-up gas. This supersonic expansion cools the sample compounds. The expanded jet is subsequently skimmed and collimated in a second vacuum chamber, where an SMB is formed. The SMB contains vibrationally cold sample compounds that pass through a dual cage EI ion source where

the sample compounds are ionized by 70 eV electrons. The formed ions are subsequently introduced into the mass analyzer to record cold-EI mass spectra.

The potential of the EI-LC-MS with SMB system was demonstrated by comparing cold EI mass spectra obtained with the EI-LC-MS with SMB system with their corresponding library EI mass spectra for thermolabile compounds such as sulfamethoxazole and haloperidol. The cold EI mass spectra shared all major fragment ions with the library mass spectra, while the molecular ion was more abundantly present, increasing the confidence level for a correct sample identification. The absence of ion suppression or enhancement effects was demonstrated by spiking a 1 ppm pyrene solution with 1000 ppm each of caffeine and ibuprofen. The flow injection analysis of "pure" and "spiked' pyrene resulted in a similar response for pyrene. This suggests that EI-LC-MS with SMB can be used without prior LC analysis, potentially enabling ultra-fast flow injection analysis.

The applicability of the EI-LC-MS with SMB system was further demonstrated for the fast-isocratic LC-MS analysis of octafluoronaphthalene, pyrene, Agidol 40 and cholesterol and the gradient analysis of dimethylphtalate, diethylphtalate, ethylparaben and butylparaben. These applications demonstrated the potential of EI-LC-MS with SMB for the analysis of relatively non-polar compounds that are hard or impossible to analyze using standard ESI or APCI based LC-MS. Relatively uniform responses were moreover obtained for the different compounds, demonstrating that the ionization efficiency of EI is independent of the compound polarity and identity.

A review on the state-of-the-art in the LC-EI-MS field was recently written by Rigano et al. Benefits and critical aspects were discussed, together with strategies to improve technological hindrances[34].

2.2. Mass Analyzers

The types of mass analyzers mainly used in pharmaceutical analysis are ion trap (IT), quadrupole (Q), Orbitrap and time-of-flight (TOF) instruments as well as a variety of hybrid instruments characterized by a high resolution, enhanced sensitivity and/or an increased mass accuracy over a wide dynamic range. Among these, the triple quadrupole (QQQ), quadrupole TOF (Q-TOF), ion trap Orbitrap (IT-Orbitrap) and quadrupole-Orbitrap (Q-Orbitrap)[35] are popular.

The performance of a mass analyzer is described by several parameters, such as the resolving power (RP), mass accuracy (MA), mass range (MR), linear dynamic range (LDR), acquisition speed and sensitivity. These parameters need to be carefully evaluated with respect to the final goal of an application. The accuracy of a measurement refers to the degree of conformity between a measured quantity and its true value. MA (in ppm) is usually calculated using the following formula: (measured mass – theoretical mass/theoretical mass) * 10^6. Internal calibration usually allows to achieve better MA values in comparison with external calibration (2-3 times more) and can be done: (i) by simultaneously introducing a sample and internal calibrant into the ion source by using a splitter tee to introduce the internal calibrant via direct infusion (ii) by using a well-defined ion with known elemental composition originating from the background[36], or (iii) by using a microfluid device with two independent ESI emitters to sequentially generate ions from different solutions for mass analysis, using a rapid modulation between ESI emitters. When using external calibration, calibrant and sample are not present in the ion source simultaneously. External calibration can provide similar results to internal calibration if the mass spectrometer is stable enough and does not present any mass drift[37] providing the time between the introduction of the sample and the calibrant is as small as possible.

The mass range (MR) is the range of *m/z* over which a mass spectrometer can detect ions. The acquisition speed is the time the mass spectrometer needs to acquire one spectrum, and is typically expressed in kDa/s for low-resolution and in Hz for high-resolution mass analyzers. Note that numerical

values of acquisition speed in kDa/s and Hz for a specific *m/z* range are identical. The resolving power (RP) refers to an instrument's ability to distinguish between two adjacent ions of equal intensity and is defined as the *m/z* value of a peak divided by its peak width, usually measured at full width half maximum (FWHM), indicated as Δ*m/z* (RP= (*m/z*)/Δ*m/z*). The RP must always be defined for a particular *m/z* value. Low, high and ultra-high resolution mass analyzers can be distinguished based on the RP, although a clear definition of the ranges is missing[37]. Low-resolution mass analyzers typically have an RP in the range of 10^3, while high- and ultra-high resolution MS have an RP in the range of 10^4-10^5 and >10^5, respectively.

Mass Resolution refers to the ability to separate two neighboring mass spectral peaks and is considered as the minimum mass difference ($m_2 - m_1$) between two mass spectral peaks such that the valley between their sum is a specific fraction of the height of the smaller individual peak. The mass resolution is the inverse of the RP expressed as *m/z* for a given *m/z* value. In general, a better mass analyzer will result in lower resolution and higher RP values.

2.2.1 Low Resolution MS

Despite their low resolving power, the development and commercialization of low-cost, small mass spectrometers (mainly quadrupole analyzers) for LC has enabled a widespread use of LC-MS in pharmaceutical applications[38,39]. Depending on the manufacturer, these mass analyzers are mainly equipped with an ESI source that can operate in positive and negative mode either in sequential runs or within a single run. Mass ranges usually vary between 10 and 1250 *m/z*, with a mass resolution between 0.5 and 0.7 FWHM[37,38]. The mass analyzers can be operated in full scan mode or selective ion monitoring (SIM) with scan rates between 3,6 and 10 kDa/s. Depending on the manufacturer, the systems can be operated at flow rates in the µL/min range (requiring a flow split before entering the MS) and even up to 1 mL/min. Despite some of their disadvantages, such as the limited sensitivity and linear range, these systems are perfectly suited for applications that require less stringent specifications. Examples can be found in reaction monitoring, where the formation of product and the evolution of starting material and intermediates generally only requires unit mass resolution; the high-throughput analysis of microplates that are used to explore a large variety of reaction conditions, and wherein a single quadrupole mass analyzer can usually provide sufficient sensitivity to monitor both starting materials and products, including reaction byproducts (Figure 2). A final example is the identification of compounds, degradation products and impurities to assure the quality of incoming raw materials and active pharmaceutical ingredients (APIs), to perform stability studies and assess metabolization[40,41]. In this type of studies, the mass analyzer ideally does not only allow quantitation, but can also help in the structure elucidation of the degradation products, impurities and metabolites.

In this perspective, it can be envisaged that these small and cheap mass spectrometers will become more prominent in routine pharmaceutical analyses, and eventually go hand-in-hand with UV detection. In more than 95% of all cases, the quantitation of pharmaceutical compounds is already performed with MS when complex matrices do not allow a reliable spectrophotometric quantification[8].

2.2.2 High Resolution MS (HRMS)

Although TOF mass analyzers have a lower mass resolution than Fourier transform (FT) mass analyzers (FTICR and Orbitrap), they virtually have no upper *m/z* limit and are thus particularly useful to identify singly charged ions of high molecular weight. Their fast response/scan rate is also advantageous for applications requiring short acquisition times, e.g., in UPLC- and or LC×LC-HRMS analysis. Orbitrap and TOF instruments typically produce a mass accuracy of 2–5 ppm. This is sufficient to assign molecular formulae to candidate molecules. It, however, becomes more difficult to identify a compound as the *m/z* of the ion increases, since more possible elemental compositions will fall within a certain mass

error, making the elemental composition assignment more difficult. Whereas HRMS was originally used in drug metabolism and metabolite identification studies due to its superior qualitative performance, it is nowadays increasingly used to perform reliable and sensitive quantitative analyses[42]. The sensitivity of HRMS is currently often comparable or superior to that of traditional triple quadrupole instruments, due to the possibility to construct extracted ion chromatograms with narrow mass extraction windows. This was for example demonstrated for the analysis of protease inhibitors, tyrosine kinase inhibitors, steroids and metanephrines in plasma samples comparing a new Orbitrap with a recent triple quadrupole[43]. Similar LOD values were reported for both MS instruments (within a ≤ 2x fold difference), whereas the upper limit of quantitation (ULOQ) and curve linearity were better for the Orbitrap (Figure 3).

2.2.3 Tandem MS

MS systems can be used in full-scan mode, providing a complete overview of the sample content (untargeted), or in targeted acquisition modes such as single ion monitoring (SIM) or extracted-ion chromatogram (EIC) mode for a single MS event. To further increase the analytical and identification capabilities of mass spectrometry, tandem mass spectrometry (MS/MS) wherein two stages of mass analysis occur in series to examine the fragmentation behavior of compounds, have found widespread use in pharmaceutical analysis[44,45]. The principle of MS/MS is that a sample is ionized and analyzed in one stage of mass spectrometry. A particular *m/z* value is selected from the mass spectrum and directed into a collision cell containing a neutral gas (in general argon, helium or nitrogen). The collision with the gas excites the ion vibrationally, a process known as collision induced dissociation (CID). Other post-source induced fragmentation approaches in the reaction chamber include electron capture and electron transfer dissociation methods (ECD and ETD), surface-induced-dissociation (SID) and photodissociation. The ions (fragments) generated by these processes are separated and recorded in a second stage of mass spectrometry. Mass spectrometers that perform the three steps of the MS/MS process (precursor ion selection, induced dissociation and mass analysis of the product ion(s)) in spatially separated devices, are called “tandem-in-space mass spectrometers”, whereas devices that execute the three steps sequentially in the same device are called “tandem-in-time mass spectrometers”. Examples of the first type are QQQ and hybrid MS instruments (Q-TOF, IT-TOF, etc), while the second category includes IT, Orbitrap, and FTICR.

Tandem MS can operate in product ion-scanning (PI) mode (MS1 in SIM or EIC mode and MS2 in scan mode); precursor ion-scanning mode (MS1 in scan mode and MS2 in SIM or EIC mode), neutral-loss scanning mode (MS1 and MS2 in scan mode), reaction monitoring mode (MS1 and MS2 in SIM or EIC mode), data-independent acquisition (DIA), and data dependent acquisition (DDA). Reaction monitoring can be executed in multiple reaction monitoring (MRM), selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) mode (Figure 4). PRM is typically executed on a high-resolution hybrid MS instrument, and unlike SRM and MRM, where one or multiple transitions are followed at a time, respectively, PRM monitors all fragments of a precursor ion in parallel[46]. This makes data acquisition methods based on PRM easier to build, since target transitions do not need to be selected in advance.

Product ion and selected reaction monitoring (SRM) scans can be used in the same method with the instrument alternating between the two different scan modes. An example is the LC-MS/MS method proposed by Mazzucchelli et al. for the analysis of doxorubicin and its major metabolite, doxorubicinol, in mouse plasma, urine and tissues. The separation of the analytes was performed in a reversed-phase LC column coupled with ESI-QQQ-MS operated in positive mode. The study was validated according to the US Food & Drug Administration (FDA) guidelines using quality control samples prepared in all matrices[47].

A common approach for a broad-spectrum drug screening is to perform an SRM scan followed by DDA of PI spectra. In DDA, a fixed number of precursor ions, whose *m/z* values were recorded in a survey scan in full scan MS, are selected based on predetermined rules and automatically subjected to a second stage of mass selection[48]. DDA allows for specific experimental conditions to be set for the collection of PI spectra of the compound of interest. This method obviates the need to first analyze the sample in full scan mode to identify target precursor ions and then re-analyze the sample in MS/MS mode to acquire MS/MS data for each of the precursors. Not all MS/MS analyzers are capable of performing these scan modes simultaneously in the same method. Recently, Fabresse and coworkers developed and validated an LC-HRMS/MS method showing the potential of DDA in the untargeted identification of new psychoactive substances with the support of available libraries[49]. After the optimization, the method was applied to 11 hair samples, allowing the detection of 284 compounds, of which 72 new psychoactive substances (including a synthetic cannabinoid (AKB48-5F) detected for the first time in hair samples).

In DIA, all ions within a specific *m/z* range are fragmented and analyzed in a second stage of tandem MS, in contrast to the sequential detection, selection and analysis of individual ions in DDA. The two most common DIA approaches make use of alternating low/high collision energies (CE) or stepwise isolation windows. The stepwise (or sequential) isolation windows approach leads to an increased specificity, which is particularly useful when analyzing more complex samples. This higher specificity, however, comes at the cost of a decreased duty cycle, depending on the size of the isolation windows and the scanned mass range, ultimately narrowing the mass range that can be sampled. The alternating low/high CE approach on the other hand leads to a higher sensitivity and is mainly limited by the sample complexity.

Examples of alternating low/high CE, in which all precursor ions are fragmented without pre-selection by the quadrupole, are MS^E and All-Ion Fragmentation (AIF). In MS^E platforms, precursor and fragment ion spectra are acquired during a single chromatographic run by alternating low and high CE scans. The low CE allow to obtain accurate mass data for the precursor ions and intensity data for quantification purposes, the high CE allow to obtain accurate masses for the product ions. In All-Ion Fragmentation, fragmentation is obtained with a higher energy collisional dissociation (HCD) cell. After the ions are trapped in the HCD cell, the energy can be increased stepwise at specific percent values around a chosen middle energy value. This is similar to the energy ramping normally employed in MS^E. Noble and coworkers[50] used DIA in MS^E mode in a targeted screening UHPLC-ESI(+)-QTOF-MS/MS method to identify 50 fentanyl analogues in blood samples without the use of reference standards, under the hypothesis that these compounds with a 4-anilidopiperidine core would follow the same fragmentation pattern as fentanyl. DIA in MS^E mode allowed all precursor ions in the ion source to be analyzed with subsequent fragmentation of the precursors induced by a ramp of CE from 10 to 40 eV, which provided a wider picture of the fragmentation pattern of each compound.

A specific variant of DIA using stepwise isolation windows is called “Sequential Window Acquisition of All Theoretical Mass Spectra” (SWATH-MS), patented by ABSciex. In SWATH-MS, pre-defined ranges of precursor *m/z* values are successively isolated and fragmented. A single precursor ion (MS1) spectrum is typically recorded, followed by a number of fragment ion (MS2) spectra with wide precursor isolation windows (e.g., 25 *m/z*). Repeated cycling of consecutive precursor isolation windows over a defined mass range, leads to a comprehensive data set that incorporates continuous information on all detectable precursor and fragment ions. In this way, extracted ion chromatograms can be generated both on MS1 and MS2 level. Arnhard and coworkers[51] compared the qualitative capabilities of DIA (SWATH) and DDA using an LC-ESI-QTOF-MS/MS for a mixture of eight compounds of pharmaceutical interest (bunitrolol, caffeine, cocaine, diazepam, doxepin, haloperidol, 3,4-

methylenedioxyamphetamine and zolpidem) at seven different concentrations (in a range of 1-1000 ng/mL). All 8 standard compounds were positively identified in DIA mode down to 10 ng/mL and 5 out of 8 also at 1 ng/mL (lowest level of concentration used), while 5 and 2 out of 8 compounds were identified by DDA at the same level of concentrations.

Although DIA data analysis is more complex than DDA, each acquisition has the potential to provide a much more complete picture of the sample and results can improve as libraries are expanded. Moreover, in DIA mode, for post-targeting purposes, EICs can be generated on MS2 as well as MS1 level, while in DDA mode it is only possible on MS1 level (in MRM it is not possible due to the targeted data acquisition). A schematic example of MRM, PRM, DDA and DIA is shown in Figure 4[52].

2.2.4. Quan/Qual analysis

In drug discovery, major applications are the quantitative analysis of the parent drug and the identification of metabolic biotransformation products. Despite the high selectivity and sensitivity of QQQ for these purposes, their major drawback is their moderate qualitative capability. Therefore, until recently, samples were often analyzed twice: a qualitative analysis by HRMS and a quantitative analysis by QQQ. HRMS in full scan mode acquires all ions present in the sample and offers a lot of qualitative information. The qualitative performance of HRMS comes from its capability to resolve ions and identify unknown molecules. Generally, unknown molecules can be subdivided into known-unknowns and unknown-unknowns. The known-unknowns are unknown to the analyst but described in literature via MS and MS^2 spectra, whereas the unknown-unknowns have not yet been identified or reported in literature[53]. HRMS can be used to identify these molecules by the accurate mass of its precursor and product ions, providing information on the relative isotopic abundance, the isotopic fine structure and ratios between product ions[54]. In combination with the improved quantitative capabilities of the new generation of HRMS systems, this has triggered analysts to switch from QQQ systems to HRMS for quantitative analysis. In fact, the integration of qualitative and quantitative analysis is of great interest in drug discovery, since sample throughput and/or volume can be critical and more high-quality data can be needed to improve the drug selection process. On a QQQ, quantitation is usually realized in SRM or MRM while in HRMS, the extracted chromatograms are only generated after the acquisition and can be simultaneously obtained with the quantitative data.

According to Kaufman et al., HRMS in full scan mode outperforms QQQ in MRM mode in terms of selectivity only if a resolution > 50,000 is achieved[55]. An example of a HRMS Quan/Qual analysis is given by the *in vivo* biotransformation study of tamoxifen by Dahmane and coworkers[56]. Plasma samples from patients treated with tamoxifen (steady-state levels) were analyzed with an Orbitrap MS (Exactive Plus) in HR-full-scan mode. Besides the targeted, quantitative determination of tamoxifen and 40 selected metabolites, HR-full-scan mode allowed to identify 39 circulating metabolites of tamoxifen, of which 3 were previously not reported. With a similar approach, Rochat and coworkers[57] discovered new metabolites of hepcidin by using HR-full-scan acquisition in the context of the quantitative determination of hepcidin.

Narrow mass extraction windows are usually used for the quantitative processing of HRMS data, instead of nominal mass product ion chromatograms (SRM or MRM). In this respect, the selection of the mass extraction window (MEW) for the construction of the extracted mass chromatogram is important. Vereyken and coworkers[58] investigated the impact of centroid and continuum data on data processing in Quan/Qual analysis by using three different HRMS platforms (Waters Q-TOF, Sciex Q-TOF and Thermo Orbitrap) coupled to the same UHPLC system. The impact on quantitative HRMS performance was evaluated using calibration curves for 8 small molecules (tolbutamide, abiraterone, norethindrone, acetaminophen, prednisone, midazolam, loperamide and simeprevir) in plasma using

four different processing softwares (Sciex Analyst® TF, Waters Masslynx, Waters Unifi and Thermo Xcalibur). The use of centroid data combined with smaller MEWs provided cleaner EICs, improved the qualitative analyses and reduced the number of false positives in targeted and untargeted analysis. The effect on the quantitative performance was furthermore minimal.

An interesting review on mass spectrometric recommendations for Quan/Qual analysis using LC-QTOF-MS was recently published by Dubbelman and coworkers[59], while a publication by Sturm et al. discusses the implementation of HRMS analysis on Q Exactive™ series MS instruments in a regulated quantitative bioanalysis environment[60].

2.3. LC-MS instrumentation

Despite the enormous developments that have been made in mass spectrometry, there are still a number of shortcomings that hamper unlocking its full potential. Firstly, (even high-resolution) mass spectrometry is unable to distinguish isomeric compounds, i.e., compounds with an identical molecular formula but a different molecular structure, that therefore often have a different biological activity. Secondly, the simultaneous introduction of multiple compounds in a mass spectrometer when dealing with complex samples, can seriously influence the ionization efficiency of the compounds of interest. This can lead to an inaccurate quantitation, but also affect the accuracy of the mass measurements. Thirdly, considering an accurate mass measurement at $m/z < 400$, even a mass error of < 1 ppm results in the generation of several potential elemental formulae, and additional tools can be required for an unequivocal identification[61]. In order to fully exploit the potential of HRMS, it is hence primordial to combine it with other separation techniques, such as liquid chromatography (LC).

Recent advances to improve the separation performance in LC, such as the development of sub-2 µm fully porous particles (FPPs) and sub-3 µm core-shell or superficially porous particles (SPPs), in combination with instrumentation capable of delivering ultra-high pressures (up to 1500 bar) and with reduced extra-column variances, are increasingly employed in pharmaceutical analysis[62–64]. Using these novel particle types in combination with UHPLC equipment, pharmaceutical analyses can be performed in less than 10 min, while the time required for method development is also drastically reduced[65]. Analysis time is typically decreased by a factor of 5−9 when a UHPLC column of 50 × 2.1 mm packed with sub-2 µm particles is used instead of a conventional HPLC column of 150 × 4.6 mm packed with 5 µm particles, while maintaining column efficiency[66]. Additionally, sub-3 µm SPP packed columns rival the efficiency of sub-2 µm FPP columns for small drugs, while only generating half the backpressure[67,68]. An evaluation of FPP and SPP columns for the analysis of drugs, including antipsychotics, antidepressants, anticonvulsants and andanxiolytics in biological samples by UHPLC-MS/MS was performed in 2017 by the Querioz group. A total of 6 columns (2 FPP and 4 SPP) with different particle sizes were compared in terms of reduced plate height curves, impedance vs reduced velocity, analysis time vs flow rate (mL min^{-1}), backpressure vs flow rate (mL min^{-1}), resolution, peak capacity, asymmetry and retention factor. Overall, the sub-2 µm columns gave the best performance for the analysis of the target drugs in plasma samples[69].

Hydrophilic interaction liquid chromatography (HILIC) has also known an important revival in the last decade and is nowadays available in a large number of stationary phases and particle designs (including the sub-2 µM FPPs and sub-3 µm SPPs discussed above). HILIC is particularly interesting in combination with mass spectrometry since the high percentage of volatile organic solvent in the mobile phase typically leads to an improved ionization efficiency and increased sensitivity. HILIC is particularly suited for the analysis of polar compounds, and in this respect is increasingly used for the analysis of polar drugs and metabolites[70,71].

These new developments in column technology aim at increasing the speed and/or efficiency of the analysis and produce narrow, highly efficient peaks. The hyphenation with mass spectrometry therefore requires state-of-the-art equipment with fast duty cycles, such as QQQ and TOF-MS. Extra-column band broadening should be minimized as well, especially when dealing with narrow-bore and short columns with reduced column volumes[72–74]. Since most UHPLC separations are carried out at flow rates between 0.5 and 1.0 mL/min, while the optimum mobile phase flow rate to achieve acceptable ionization yields and sensitivity in ESI is rather between 0.05 and 0.5 mL/min, some technical modifications have been proposed. These include the utilization of additional heated gases to focus the nebulizer spray and improve ion desolvation and modifications to the ion source configuration[75].

A fast (5 min) UHPLC-UV-ESI(+)-QTOF-MS method using a C18 sub-2 µm FPP column was developed and validated by Schreiber and coworkers for the separation and determination of tadalafil (a drug used for erectile dysfunction) and its impurities in pharmaceutical samples. The proposed method was validated according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use in terms of accuracy, selectivity/specificity, linearity and robustness[76]. A UHPLC-MS/MS method for the simultaneous determination of phenacetin, omeprazole, metoprolol, midazolam and their metabolites in rat plasma using a short C18 50 × 2.1 mm sub-2 µm FPP column coupled to an ESI(+)-QQQ-MS operating in MRM mode was developed and applied by Wang and coworkers. After liquid-liquid extraction of the plasma samples, analytes were characterized in less than 2 min and the method was validated for accuracy, precision, matrix effects and linearity[77]. Rakuša and coworkers used a short C18 50 × 2.1 mm sub-3 µm SPP coupled to an ESI(+)-QQQ-MS operating in MRM mode for the fast analysis of rifampicin in human plasma after protein precipitation and solid-phase extraction. The total run time including re-equilibration was 2.4 min. After the optimization and validation according to EMA guidelines, the method was used to quantify rifampicin in plasma samples of 340 patients, demonstrating that it is suitable both for therapeutic drug monitoring and pharmacokinetic analysis[78]. Core-shell technology was also employed for the separation of total and free daptomycin (antibiotic drug) in human plasma. Free daptomycin in plasma was obtained by centrifugal ultrafiltration. Deproteinized plasma samples were directly separated on a C18 150 × 2.1 mm sub-3 µm SPP column under isocratic elution conditions in 10 min. The RPLC column was coupled with an ESI(+)-QQQ-MS operating in MRM mode for detection and quantification[79].

For complex samples, containing a mixture of compounds with a large variety in physicochemical properties (e.g., containing both very polar and very apolar compounds), even the most efficient columns can often not resolve all important peaks in the chromatogram[80,81]. In this case, two-dimensional liquid chromatography (2D-LC) techniques can be employed to increase the separation capacity. Since commercially available 2D-LC systems have recently entered the market [82,83], 2D-LC approaches are nowadays also increasingly employed in pharmaceutical analysis.

Two-dimensional separations are predominantly performed in heart-cutting (LC-LC) or comprehensive (LC × LC) mode[84]. In heart-cutting mode, the effluent of a first-dimension separation is only collected in a region of particular interest (e.g., where compounds are co-eluting) and re-injected on a second-dimension column for further separation. Typical applications of heart-cutting 2D-LC in pharmaceutical analysis involve the isolation of impurities from the active pharmaceutical ingredient (API) or an interfering matrix in the first dimension, the combination of achiral and chiral columns for the separation of enantiomers in the second dimension, and online desalting of peaks eluting in MS-incompatible mobile phases in the first dimension[83,85–87].

Online heart-cutting 2D-LC was for example applied to identify an unknown impurity that was formed when an API was stored in an IV delivery device in the presence of polyethylene glycol (PEG)[85]. This impurity co-eluted with PEG, but could clearly be detected in UV due to the lack of UV-absorbance of PEG. In MS, however, it was completely obscured by PEG oligomer peaks, making identification of the impurity impossible even when using high-resolution Orbitrap MS. Online 2D-LC/MS was therefore applied to separate the impurity from the PEG matrix. Both first- and second-dimension separations were done on C18 columns. The first-dimension separation was carried out on a FPP 5 µm column employing mobile phases consisting of trifluoracetic acid (TFA) in water and acetonitrile. The second-dimension separation was carried out on a SPP 2.7 µm column and employed formic acid in water and acetonitrile as mobile phases. These conditions were selective enough to separate the impurity peak from the PEG matrix to gain sufficient MS abundance for structure elucidation. Another application of online heart-cutting 2D-LC-MS involved the combination of achiral and chiral columns to evaluate the brain response of a chiral compound in rat brain microdialysates[86]. The achiral column was used to evaluate the metabolization of the chiral compound (by separating its main metabolites) and its biological activity (by separating the neurotransmitters dopamine, acetylcholine and 5-hydroxytryptamine). The chiral compound itself, which eluted last from the achiral column, was heart-cut and transferred to a chiral column to measure the brain concentration of the single enantiomers in order to investigate potential stereoselective adsorption or preferential metabolism of one of the enantiomers.

Many pharmaceutical analyses still require mobile phases that are incompatible with MS (containing ion-pairing reagents or non-volatile salts) to separate the API from its impurities. 5-aminolevulinic acid (ALA) for example, is a very hydrophilic compound that cannot be retained or separated from its impurities under standard reversed-phase conditions. The addition of ion-pairing reagents to the mobile phase leads to sufficient retention of all compounds but makes the method incompatible with MS, which is essential for the identification of unknown impurities. Therefore, an online heart-cutting method was proposed wherein ALA and its impurities were first separated on a C18 column using a mixture of sodium octanesulfonate/potassium dihydrogenphosphate/acetonitrile as the mobile phase[88]. Each impurity and the main compound were subsequently trapped individually in a trap column (each time requiring a separate injection) and transferred to the second-dimension column, which consisted of a C8 column and was operated using a mobile phase consisting of water, acetic acid and acetonitrile. The eluent of the C8 column was finally eluted to an ESI-MS/MS system for structure elucidation. By diverting the eluens of the C8 column to the waste for the first minute of the analysis, contamination of the MS by non-volatile salts was avoided and ion suppression significantly reduced.

Online heart-cutting generally only allows the analysis of a very limited number of fractions eluting from the first-dimension column, since a new fraction can only be analyzed once the previous fraction has been separated on the second-dimension column. As demonstrated by the previous example, this might require the same sample to be injected multiple times, each time fractionating another impurity. One way around this is to apply multiple heart-cutting 2D-LC. In this approach, a multitude of heart-cuts from the first dimension separation are sampled into multiple loops or trap columns, where they remain parked until they are analyzed on the second dimension column[82]. By parking the fractions eluting from the first-dimension separation in this way, the timescales applied in the first and second dimension are disconnected. This allows optimizing the separation conditions in both dimensions independent of each other to obtain the maximum possible resolution in both dimensions. Since multiple fractions can be taken from the first-dimension separation at once, the time required to identify all impurities in a sample is significantly reduced compared to “single” heart-cutting 2D-LC.

Multiple heart-cutting 2D-LC was for example applied to characterize eight impurities in cefpiramide[89]. For this purpose, cefpiramide was first separated from its impurities using the non-volatile mobile phase conditions prescribed by the US Pharmacopoeia in the first dimension. Each peak separated on the first-dimension column was subsequently trapped into a separate 20 µL loop and transferred to a second-dimension column once the first-dimension separation was complete. Employing a volatile mobile phase for the second-dimension analyses, the peaks could be eluted towards an IT-TOF-MS equipped with ESI interface for detection and structure elucidation. In 2019, the same research group applied a similar multiple heart-cutting 2D-LC-ESI-IT-TOF-MS method for the separation and characterization of new components and impurities in leucomycin[90]. A non-volatile buffer solution was used as the mobile phase in the first-dimension, modifying the gradient of the non-volatile mobile phase method reported in the Chinese and Japanese Pharmacopoeia for leucomycin to improve the separation. Peaks eluting from the first-dimension were trapped using a switching valve and sent to the second-dimension column and then to the MS system using a volatile mobile phase consisting of methanol and ammonium acetate for the second-dimension. Six impurities were previously not reported. Four of these impurities were acid degradation products, while the other two impurities were process impurities.

Another particular form of heart-cutting was proposed to increase the sensitivity of the analysis of midazolam and 1'-hydroxymidazolam in plasma[91]. For this purpose, 80 µL of protein precipitated plasma was first injected on a 2.1 mm I.D. trapping column. The trapping column was subsequently switched in-line with a 1.0 mm I.D. UHPLC column to separate the sample. When the analytes of interest eluted, the 1.0 mm I.D. column was switched in-line with a second trap column with an I.D. of 0.5 mm to trap the heart-cut. Finally, the second trap column was back-flushed onto a chip-based column with an I.D. of 0.15 mm and an orthogonal separation was performed. By gradually decreasing the column diameter along the flow path, the high sampling efficiency (i.e., efficiency of ion creation x ion transfer from atmosphere to vacuum) obtained when coupling a low I.D. column operated at a low flow rate with an ESI-MS, could be combined with the high sample loading capacity of a large I.D. column. This resulted in an increase in sensitivity that was 250-500 x higher compared to direct injection of 0.2 µL of the same sample on the chip-based column, and 10-50 x higher compared to injecting 10 µL of the same sample on the chip-based column after trapping on a trap column.

Another 2D-LC application was proposed by Yu et al. using an LC-LC-ESI-QQQ-MS/MS method for the determination of tramadol and its phase I and II metabolites in hair. After extraction from hair, drugs were analyzed using a HILIC column in the first dimension for class separation and a RPLC column in the second dimension for intra-class separation. Identification and quantification of the analytes was done in selected reaction monitoring (SRM) mode. The method was validated in terms of LOD, LOQ, accuracy, sensitivity, and matrix effects[92].

In comprehensive 2D-LC (LC×LC), the entire effluent eluting from the first-dimension column is fractionated and transferred to the second-dimension column for further separation. By applying a very short sampling time (modulation time, usually less than 2 min), each peak eluting from the first dimension is sampled at least three or four times. This generally requires a very slow first dimension separation in combination with a very fast second dimension separation to avoid losing the resolution obtained in the first dimension. LC×LC leads to very powerful separations, but also to complex data since every compound is cut into multiple peaks. Choosing the two separation dimensions can be critical and is not straightforward. Particular attention should be paid to the complementarity of the stationary phases, the miscibility of the mobile phases used in the first and second dimension and the impact of the injection of the first-dimension mobile phase on the separation in the second dimension. A comprehensive overview about possible combinations in retention mechanisms (selectivities) in

LC×LC has recently been given by Pirok et al., evaluating potential peak capacities, column re-equilibration times and other critical requirements mentioned above[93]. Since comprehensive 2D-LC provides a global perspective on the sample, it is often used for very complex samples typically encountered in proteomics, metabolomics, biopharmaceutical analysis and natural products [94–96].

Examples of comprehensive 2D-LC in combination with MS for pharmaceutical analysis were recently given by Heinisch et al.[97,98]. In their studies, the authors defined generic 2D-LC systems that can be used to improve the knowledge on impurities during drug development significantly. For this purpose, the degree of orthogonality and the practical peak capacity of 190 RPLC x RPLC configurations was first assessed for two real pharmaceutical samples[97]. Out of these 190 combinations, three RPLC x RPLC configurations with the same second dimension were selected that provided i) a very high peak capacity for the first configuration, ii) a sufficient degree of orthogonality between the first and second dimension for the other two configurations, iii) a second-dimension column capable of withstanding high temperatures and iv) second dimension conditions that were MS compatible. These three configurations were hyphenated to a UV and a single quad MS to identify impurities in two pharmaceutical samples. Figure 5 shows the utility of MS to identify impurities by comparing the second-dimension chromatograms obtained with UV (Fig. 3a) and MS (Fig. 3b) for the pharmaceutical samples. Two compounds (indicated in red in Fig. 3b) were only detected with MS. For all three configurations, peak capacities close to 900 were obtained in less than 50 min, while most impurities could be detected with each of the three configurations. While the use of one LCxLC – UV/MS system outperformed the number of compounds detected by one 1D-LC-UV/MS system with 40%, the addition of a second and a third LC×LC – UV/MS system further increased this number by 12% and 3%, respectively. These findings suggest that a limited number of well-chosen combinations of LC systems can be sufficient to characterize any pharmaceutical sample. In 2019, the same research group assessed the quantitative performance of an online RPLC × RPLC system for pharmaceutical analysis, taking into account the low quantitation and detection limits required by ICH guidelines[98]. An in-house developed procedure, using predictive calculation tools and a Pareto optimization approach, was used to optimize the RPLC × RPLC conditions. Similar to the previous publication, two sets of conditions were selected: one favoring a high effective peak capacity and one favoring a low dilution factor (Set-up #1 and Set-up #2, respectively). The optimized RPLC × RPLC separations resulted in peak intensities that were 3-4 fold higher with Set-up #2 in comparison with Set-up #1 for low level impurities. Set-up #2 allowed to detect impurities with LOQs between 0.05% and 0.1% of the API. The optimized conditions were then applied to analyze a real pharmaceutical sample. Impurities present at concentrations as low as 0.04% of the API were detected with S/N ratios above 3, whereas impurities at concentrations of 0.1% of the API were detected with S/N ratios above 10. A recent overview of studies and emerging trends in online 2D-LC techniques applied to pharmaceutical compounds, covering the late 1980s to 2017, is given by Iguiniz & Heinisch[99].

2.4. Miniaturization and Microfluidics

Capillary and nano-scale LC in combination with MS have the ability to address small sample sizes and provide high sensitivity information, which is particularly interesting when dealing with small sample volumes and low abundant compounds. When column dimensions are moreover reduced to 100 µm or less, there is no need for a nebulizer gas, as long as the mobile phase contains a reasonable concentration of organic modifier (15-20%)[100]. Originally, capillary and nano-scale LC were mainly used in proteomics, since in drug development, drug metabolism and pharmacokinetic (DMPK) studies, there is rarely a limitation in sample volume. The high level of sensitivity achievable with capillary and nano-scale LC can, however, also be useful in pharmaceutical applications, for example to create an entire pharmacokinetic (PK) profile from a single animal, since only small sample volumes are

required[101]. The analytical method should, however, be sufficiently robust to ensure the entire batch of samples can be analyzed using the same set-up. The extra sensitivity of capillary scale separations can also be used to identify metabolites in very early *in vivo* pharmacodynamics studies requiring only minimal amounts of the candidate drug. This allows the compound to be fully evaluated without needing larger scale synthesis[102].

Capillary and nano-scale separations, however, also have a number of drawbacks. Due to the small column volumes and difficulty of making dispersion-free connections, extra-column band broadening is more of an issue than in normal bore separations. The small ID columns and tubings are more prone to blockage, especially when dealing with "dirty" samples. And thirdly, there is a need for MS sources that can effectively work with LC flows in the nL-µL range. To overcome some of these issues, fully integrated microfluidic devices or chips have been developed. These devices commonly combine an open tubular LC column with reduced column ID with improved spray/tip connections for hyphenation with MS. A recent study comparing four emitter geometries for the hyphenation of LC microchips to MS demonstrated that at flow rates down to 15 nL/min, a perfectly three-dimensional sharp ("pulled") emitter tip performed best. However, at flow rates above 50 nL/min, less sharp (ground pyramidal shaped) or corner cut and even blunt emitters performed equally well[103]. Schematic drawings and SEM images of these emitter geometries can be found in Figure 6. These findings indicate that elaborate techniques to sharpen or ground electrospray emitter tips are less critical in chip-based LC-MS operations, compared to low flow rate techniques such as chip electrophoresis.

Microchips furthermore can also incorporate other components, such as trap or enrichment columns and valves. By combining multiple components on a single chip, void volumes are drastically reduced. Challenges that remain are the limited loading capacity, that can however at least partially be relieved by a pre-concentration step, and potential clogging problems.

The potential of a capillary scale (300 µm ID) ceramic microfluidic LC device in combination with MS/MS for DMPK studies employing low-volume biological samples has been demonstrated by Nicholson *et al*[104]. In comparison with a standard 2.1 mm ID UHPLC column, the capillary ceramic microfluidic column resulted in an 11- to 38-fold increase in sensitivity for a broad range of pharmaceuticals when employing the same triple quadrupole mass spectrometer and an identical injection volume of 1 µL. This is slightly lower than the theoretically expected increase of 49, but nevertheless demonstrates the potential of the microfluidic device for the analysis of pharmaceuticals in small sample volumes, such as those obtained from dried blood spots or other blood microsampling techniques. The loading capacity of the ceramic microfluidic device was also investigated and it was demonstrated that, in combination with a reversed-phase gradient, an aqueous solution of the antidepressant alprazolam with a volume up to 2 µL could be injected onto the column without negatively affecting the chromatographic performance. For a biological sample spiked with alprazolam and submitted to protein precipitation and therefore contained in a large percentage of acetonitrile (~ 60%), the maximum injection volume was 1.2 µL. Note that these results are very analyte dependent (depending on the retention on the analyte) and will also depend on the length of the column and the injection mode. The robustness of the ceramic microfluidic LC-MS system towards the injection of plasma-derived samples was demonstrated for 1000 sample analyses, while the intercolumn performance of six different microfluidic devices was demonstrated in terms of reproducibility and column efficiency.

Several companies (Agilent Technologies, Eksigent, Waters) have meanwhile commercialized miniaturized LC systems with integrated LC microchips for chip-based LC-MS analysis[105]. Depending on the manufacturer, these microchips are available in different channel lengths (up to 150 mm), particle sizes (ranging between sub-2 µm, 3 or 5 µm fully porous, and 2.7 µm superficially porous particles) and

stationary phases (C18, C8, graphitized carbon and HILIC). Steuer and coworkers compared a conventional LC-MS/MS set-up with an Eksigent microflow (MF) LC-MS/MS for the simultaneous quantification of 40 antidepressants and neuroleptics in whole blood within the framework of full method validation. LC analyses were carried out on a Polar RP 2.5 µm FPP column (100 x 2.0 mm) connected with a linear ion trap quadrupole mass spectrometer (ABSciex 5500 QTrap), while MFLC analyses were performed on a 2.7 µm Phenyl-Hexyl SPP micro-column (50 x 0.5 mm) connected with a linear ITQ-MS equipped with an ESI interface (ABSciex 4500 QTrap). Both mass spectrometers have the same geometry, ion source and scan speed, but the 5500 system has a larger ion inlet diameter, leading to a slightly higher sensitivity. It should furthermore be noted that different gradient profiles and injection volumes were used on both columns (10 µL on the conventional column and 5 µL on the MFLC). The authors demonstrated a dramatically lower solvent consumption for the MFLC system, and an improved peak separation (no further explanation was however provided for the choice in gradient profile on each of the chromatographic systems, or how these gradient profiles were obtained). In terms of peak interferences, LOD, MEs and precision, the systems were shown to perform comparably under the applied conditions. Advantages of the LC system were the linear calibration models and more stable retention times, leading to a higher robustness, while the system was also easier to maintain in terms of column care and column life time[106].

The Agilent Chip Cube was used in combination with a Q-TOF MS to examine the composition of Ukrain, a drug used for the treatment of different types of cancer[107]. Ukrain is a semi-synthetic alkaloid derivative of *Chelidonium majus*, a medicinal plant of the *Papaveraceae* family. To unravel the compoisition of Ukrain, TLC, semi-preparative TLC and Chip Cube LC-MS/MS experiments were conducted on Ukrain samples and compared with an alkaloid extract of *C. majus* roots. It was demonstrated that Ukrain contains a mixture of alkaloids. Some of them, such as stylopine, norchelidonine and hydroberberine, were reported for the first time.

The Chip Cube was hyphenated with ICP-MS for the analysis of cyanocobalamin in equine plasma as a means to distinguish inorganic cobalt and cobalamin in doping investigations[108]. The use of a nano LC flow allowed to reduce the organic solvent load introduced into the ICP-MS, reducing signal suppression. To hyphenate the Chip Cube to ICP-MS, the electrospray tip was removed and the eluent diverted to an unused stator port in the chip, prior to introduction into the nebulizer. The nebulizer was modified to reduce post-column band broadening, while the spray chamber was adapted to introduce a make-up gas flow at an angle perpendicular to the nebulizer flow. This prevented the nebulized eluent from depositing on the spray chamber walls, since no carbon deposition on the cones or baseline drift was observed during six weeks of operation. The developed method displayed good linearity of 0.9999 between 10 and 1000 ng/mL and a limit of detection of 14 ng/ mL (corresponding with 0.57 ng/mL Co).

Houbart et al. reported a chip-based LC MS/MS method for the quantification of fluoxetine and its metabolite (norfluoxetine) in serum samples of rat. A 20 µL serum sample was vortexed, centrifuged and then subjected to SPE before the chip-based LC-MS analysis. Linearity was obtained in the range of 25–500 ng/mL for both compounds. The LODs of fluoxetine and norfluoxetine were 0.18 ng/mL and 0.67 ng/mL, respectively. The precision (RSD) of the LC-MS/MS method was 3.0–13.3% and 6.4–16.5% for fluoxetine and norfluoxetine, respectively[109].

LC chip MS systems are expected to have more widespread applications in the pharmaceutical field in the near future. From a high-throughput point-of-view, chip-size LC-MS devices can lead to a reduction of the total analysis time, increasing the number of possible runs per day. Alternatively, these devices may in the future be used in applications such as tissue engineering and organ-on-chip platforms to monitor cells and tissues in real-time during their development and in response to drugs[110].

2.5. Matrix Effects

Despite the high specificity of LC-MS techniques for drug analysis, an adequate sample preparation step to isolate the drug and its metabolites from the matrix remains necessary in many cases. An ineffective separation can result from interactions between sample impurities and the stationary phase, increasing the noise level at the detector; interaction of the drug of interest with other matrix components and poor resolution. All these shortcomings lead to so-called matrix effects (MEs), that severely limit quantitative analysis and affect the reproducibility, linearity, and accuracy of the methods. MEs can be regarded as an increase (ion enhancement) or loss (ion suppression) in response and are often caused by an altered ionization efficiency of compounds of interest due to co-eluting analytes in the same matrix[111].

Different strategies can be applied to minimize MEs, such as different sample preparation approaches, dedicated chromatographic techniques, the application of appropriate calibration methods, and the choice of the correct ionization source before the MS detection. Widespread pretreatment methods in the pharmaceutical field are solid phase extraction (SPE) and liquid-liquid extraction (LLE), while supported liquid extraction (SLE) and solid-phase microextraction (SMPE) are also more and more commonly applied. Several studies demonstrate that a major contribution to MEs in biological fluids can be ascribed to glycerophosphocholine (GPCho) lipids due to their surfactant-like properties[112,113]. Yan and co-workers used a LLE method based on ethyl acetate to remove glycerophosphocholines from biological samples for the determination of ropinirole in rat, eliminating the matrix effect[114]. Koller and coworkers used a phospholipid-removal microelution-solid phase extraction method (µSPE) to eliminate the matrix effect in human plasma samples for the simultaneous determination of aripiprazole, its metabolite dehydro-aripiprazole, olanzapine, risperidone, paliperidone, quetiapine, clozapine and caffeine[115].

SPE has several advantages compared to LLE techniques. It can be easily automated, and can be performed on-line in combination with LC. A wide range of SPE sorbents are available and a large number of protocols can be conceived to selectively purify the compound of interest from interferences. Such sorbents include surfactant-modified sorbents, mixed-mode polymeric sorbents, molecular recognition sorbents (immunosorbents, moleculary-imprinted sorbents, aptamers) and nanostructure materials (carbon nanotubes, electrospun nanofibers)[116]. Different approaches exist to automate both off-line and on-line SPE. Precisions are usually similar for both techniques, whereas recoveries for SPE are in general better than those for LLE[117]. An example of off-line and on-line SPE-LC-MS/MS was reported by Soichot et al., for the detection of opioids in human urine samples. Analyses were carried out in urine samples by offline SPE followed by an LC-MS targeted screening for common opiates. The concentrations of morphine and codeine were also determined in plasma samples using automated online SPE-LC–MS/MS. The separation of the analytes and the internal standard was carried out using an RPLC column and detection was done using ESI(+)-QQQ-MS in MRM mode. The method was validated for specificity, accuracy, precision, stability, linearity, carry-over, matrix effects and recovery according to the European Medicines Agency (EMA) guidelines[118].

Improving the chromatographic separation can also help to overcome MEs, by separating the compounds of interest from the matrix to avoid mutual interactions and competitions. Different stationary phases, mobile phases and gradient conditions can be applied to change the retention times of components in a complex mixture. As discussed in Section 2.3, several recent column and separation strategies allow achieving a better resolution and reduce MEs. Finally, also the MS conditions can be changed to reduce MEs. It is well known that the intensity of MEs depends on the ionization technique applied, the source design or positive/negative ion acquisition. Among the various API techniques, ESI is more sensitive to MEs than APCI, while APPI is the least sensitive[27].

Besides the aforementioned strategies to limited MEs, a suitable calibration technique is also indispensable to compensate for changes in signal. The standard addition method is probably the most effective way to compensate for MEs on the method performance[119]. However, this approach is laborious and time-consuming[120]. The external standard calibration method is not appropriate if standards are spiked in a solvent instead of in the matrix, since MEs will not be adequately accounted for in a solvent. The most effective calibration approach to minimize MEs is through the use of a structural or stable isotope labelled (SIL) internal standard (IS), where the analyte of interest and the IS should undergo the same procedure(s). Recently, Whang and coworkers developed a multi-matrix LC-MS/MS method for the determination of urea in plasma in the context of human respiratory diseases[121]. Two stable-isotope-labelled urea isotopologues, [$^{15}N_2$]-urea and [^{13}C,$^{15}N_2$]-urea, were used as the surrogate analyte and the internal standard, respectively, providing the best measurement consistency across different matrices. An alternative approach is the use of stable isotope labelled reagent molecules to derivatize compounds of interest (for example to make them more ionizable under ESI-MS) for differential analysis in two comparative groups of samples (e.g., patients under drug treatment and a control group). This approach is called stable isotope-coded derivatization (ICD) and has been used as a method to correct for run-to-run ionization differences, including matrix effects[122]. Higashi et al. developed a LC-ESI-QQQ-MS/MS method for a differential analysis and quantification of allopregnanolene and its precursor pregnenolone (neuro-steroids) in brain rat after converting them to highly detectable derivatives using 2-hydrazino-1-methylpyridine (HMP) and its isotope-coded reagent ($^2H_3$–HMP (*d*-HMP))[123].

2.6. Ion mobility mass spectrometry

The principle of ion mobility is based on the drag force the ions of interest experience when moving through an electrical field in a drift cell, while at the same time undergoing collisions with stationary buffer gas molecules (typically nitrogen or helium gas) in the cell. Different forms of ion mobility based on this principle exist, such as Drift-Tube ion mobility (DT-IM), Travelling-Wave ion mobility (TW-IM) and Trapped ion mobility (TIM), and are currently available in integrated commercial LC-MS instruments[37]. In DT-IM, for example, the ions move in a linear drift tube through a homogeneous, continuous electric field filled with a neutral gas. The combination of acceleration due to the electric field and deceleration due to collisions with the gas leads to a state of equilibrium wherein the molecules move at a constant velocity. This velocity depends on the applied electric field and structural characteristics of the molecules, such as size, shape, electrical charge and mass (their collision cross section or CCS). In this way, IM allows separating isomers, isobars, and conformers, reducing chemical noise and measuring ion sizes. Additionally, ions of the same charge state and structurally similar ions can be separated in ion families which appear along a unique mass-mobility correlation line[124].

The main field of application of LC-IM-MS is the analysis of complex samples such as in proteomics and metabolomics and only few applications have been developed in the context of small molecules in pharmaceutical analysis. Recently, Hernández-Mesa and coworkers characterized phase II steroid metabolites (*i.e.*, androgen and estrogen conjugates, including glucuronide and sulfate compounds) in urine samples using an LC-IM-TOF-MS system. It was observed that the S/N ratio improved 2-7 fold when IM was combined with LC-MS. Besides separating isomeric steroid pairs (etiocholanolone glucuronide and epiandrosterone glucuronide, 19-noretiocholanolone glucuronide and 19-norandrosterone glucuronide), the IM dimension also allowed to separate steroid-based ions from co-eluting matrix compounds with similar *m/z* ratios[125].

The addition of IM to LC–MS workflows in pharmaceutical analysis can also bring an improved spectral quality to DIA modes by filtering MS and MS/MS data according to drift time in addition to retention time, by the increased duty cycle, by separating ions of different charge states and the potential to

separate isomeric and isobaric compounds. Experimentally determined CCS values can moreover be used to obtain conformational information about the compound of interest and can be used as an additional identification parameter.

3. Applications of LC-MS in Pharmaceutical Analysis

3.1. Drug Metabolism Studies

At the discovery stage, it is not only necessary to qualitatively identify metabolites in *in vitro* and *in vivo* studies, but it is also important to quantitatively estimate the importance of each metabolite so that metabolic liabilities can be addressed during compound optimization. This means that major pharmacologically active metabolites must be identified and if necessary monitored in discovery and investigational new drug-enabling toxicological studies. Early phase metabolism studies typically rely on the use of LC-MS to analyze samples derived from *in vitro* incubations (e.g. with liver microsomes or hepatocytes) and/or *in vivo* studies. LC-MS allows the rapid identification and partial structural characterization of metabolites, with a high sensitivity for most drug candidates and metabolites[126].

The metabolism of LDN-193189 (a drug that was shown to be effective in a mouse fibrodysplasia ossificans progressiva (FOP) disease model after oral administration) was investigated by Padilha and coworkers in liver microsomes and cytosol fractions of animals and human. Metabolic profiling and characterization of LDN-193189 and metabolites were performed using an RPLC-ESI-LIT-MS system in DDA positive mode[127]. Chavan et al. characterized the metabolic profile of pabociclib (PAB) *in vivo* and *in vitro*, identifying 14 metabolites in *in vivo* matrices (urine, faeces and plasma samples of rats after oral administration of PAB) and few metabolites *in vitro* (rat microsomes). SPE was applied for protein precipitation, while metabolite identification and characterization were performed using UHPLC-ESI-QTOF-MS/MS[128]. Pierre et al. monitored the change of the serum metabolite profile of guinea pigs induced by cisplatin and its association with ototoxicity. Blood samples were collected 4 days after the drug treatment and the polar metabolome was analyzed by UPLC-(+)ESI-QTOF. Results showed that cisplatin treatment changed the metabolite profile significantly and similarly in both groups (one with pretreatment with sodium thiosulfate as otoprotector and one with sodium chloride), showing that the otoprotecting effect of a pretreatment with sodium thiosulfate was very modest[129]. Al-Ghobashy and coworkers developed and validated an SPE-RPLC–ESI-QQQ-MS/MS method according to FDA guidelines for the simultaneous determination of some anticancer drugs such as methotrexate, 6-mercaptopurine and its active metabolite 6-thioguanine in plasma of children with acutelymphoblastic leukemia, correlating the results obtained with genetic polymorphism[130].

Szultka-Młyńska and coworkers combined electrochemical (EC) oxidation and MS identification to monitor the oxidation pathway of ten cardiovascular drugs. Oxidation was obtained in an EC thin-layer cell coupled online with ESI-QQQ-MS and further characterization of electrochemical products was carried out on an off-line EC RPLC-ESI-QQQ-MS. *In vivo* experiments were also executed by administering selected cardiovascular drugs to patients and analyzing their plasma and urine samples. The *in vivo* and electrochemical study resulted in comparable findings[131].

3.2. High-Throughput LC-MS for drug discovery

The throughput of an analysis can easily be increased, without significantly modifying the experimental conditions, by using multiple columns in parallel. In this case, the actual analysis is performed in a first column, while a second one is being equilibrated. Alternatively, multiple samples can be injected in series. This significantly increases the number of samples that can be analyzed per day, but also leads to a high solvent consumption and high costs. This approach is called “staggered”, “multiplexed” or

"MISER" (Multiple Injections in a Single Experimental Run) chromatography. Currently, several manufacturers offer staggered systems, such as MPX (Sciex), Transcend II (Thermo) and StreamSelect (Agilent). Since chromatographic separations in the sub-5 s range can nowadays be carried out, faster autosamplers are needed to further improve the speed of analysis in high-throughput LC–MS analysis. Zawatzky and co-workers developed a dual needle autosampler increasing the throughput of MISER analysis, enabling a 10 s injection cycle time using an UV-ESI-QMS (Figure 7). The entire analysis of a 96-well microplate was performed in 17 min. In addition, this autosampler could be installed onto any standard HPLC system[132].

Analysis times lower than 2–5 min for relatively simple samples allow to process large sample pipelines in a fast and reliable way uninterrupted in 24/7 routine operations in drug research and development. This makes UHPLC-MS very attractive for combinatorial synthesis monitoring or drug metabolism and pharmacokinetics studies (DMPK). When dealing with such short analysis times, the gradient delay volume (the sum of all volume contributions from the point of gradient formation to the column head) of a UHPLC system becomes critical for the overall sample throughput. A small gradient delay volume is advantageous in terms of analysis time, since it will ensure the changing eluent composition reaches the head of the column in a timely way. The gradient delay volume is composed of the mixer volume, the sample loop, other fluidic parts of the autosampler and all connection tubing. Although fast LC separations < 2 min can be achieved, it is important to keep in mind that the total cycling time is usually 2-3 times longer than the separation time due to the column equilibration, potential wash needle cycle, and the time needed for preparing the sample injection[133]. Chounchman and coworkers used a 5.0 mm core-shell column for the ultra-rapid LC-MS/MS analysis of clozapine and norclozapine in human plasma in gradient mode[134]. The short total analysis time of 36 s made this approach extremely well-suited for therapeutic drug monitoring, where the rapid availability of results allowed for fast dose adjustments. The combination of an ultra-short column containing core-shell particles with a high flow rate (2 mL/min) allowed a very short reconditioning time (16.4 s). Target compounds were detected on a triple quadrupole MS in MRM with a dwell time of 3 ms per transition, which allowed acquiring an average of 15 data points per chromatographic peak. In comparison with flow-injection analysis, the analysis time was only slightly increased, while matrix effects were decreased due to the chromatographic separation. Hettiarachchi and coworkers described the isolation of small molecule and biomolecule crude mixtures at the microgram-scale (100–2500 g) in 1 min using an UHPLC-PDA-ESI(+)-QMS set-up equipped with small-particle (1.8–3.5 µm), 50 × 4.6 mm analytical C18 columns and optimized flow rates (0.75–1.5 mL/min) with pressures averaging 10,000 psi[135]. Recently, Xiang and coworkers used a 2.7 cm (effective length), narrow open tubular (NOT) coated C18 column for an ultra-fast gradient LC separation of 6 amino acids in less than 700 ms at a pressure of 20 MPa using a fluorescence detector. According to the authors of that work, ultrafast NOT-LC will play an important role in high-speed and high-throughput pharmaceutical analyses, once NOT-LC will be coupled with MS (currently in development)[136].

3.3. Analysis and identification of impurities and degradation products

Impurities in pharmaceutical products are components that are responsible for changes in quality with respect to safety and efficacy. Sources of impurities include starting materials, reagents, catalysts, intermediates, solvents and degradation products formed during storage of the drug. Impurities are classified into categories depending on their origin, composition and biological safety[137]. Potential genotoxic impurities (PGIs) and genotoxic impurities (GIs) or mutagenic impurities (MIs) include

compounds that result from chemical synthesis or degradation, and are DNA reactive and can hence cause DNA damage and mutations. In the process of their identification and control, impurities are first classified according to their mutagenic and carcinogenic potential[138]. LC–MS is considered a mainstay tool for their structural characterization. Critical practical aspects for the characterization of impurities and degradation products by LC-MS were reported in a review by Narayanam et al. in 2014[139]. Dousa et al. reported the HILIC−ESI(+)-QMS determination of GIs of 2-chloro-N-(2-chloroethyl)-ethanamine in the vortioxetine (drug used to treat major depressive disorder) manufacturing process. GIs were quantified using the SIM acquisition mode at the 75 ppm level with respect to vortioxetine and the method was developed and validated according to the requirements of regulatory agencies[140]. Shelke and coworkers recently reviewed the trends in analytical methods used for degradant, foreign matter, genotoxic impurity and impurity profiling studies during the years 2013–2017[141]. Recently, Guo and coworkers studied the degradation behavior of sutezolid (oxazolidinone anti-TB compound used for the treatment of *M. Tuberculosis*) under different conditions, identifying 4 impurities by LC-ESI(+)-QTOF-MS/MS. For the structure determination of the impurities, NMR analyses were also performed to support LC-MS identification[142]. Guvvala et al. used a similar analytical setup (LC-ESI-QTOF-MS and NMR) to determine six degradation products of cangrelor (antiplatelet parenteral drug) after it was subjected to acid, base and peroxide stress conditions[143].

3.4. Analysis of chiral impurities

Chiral impurities are organic impurities present in drug substances, with a similar structure, but a different spatial orientation around a chiral carbon atom in the molecule. Since many drugs are chiral, increasing efforts are made by pharmaceutical companies to develop improved and well-controlled enantioselective manufacturing processes. Drug substances with optical isomers typically have chiral impurities. These impurities can have a different therapeutic and pharmacological profile compared to the drug substance and can be toxic in some cases (e.g., the *S*-isomer of thalidomide is a teratogenic impurity which causes birth defects[144]). Therefore, these impurities should be controlled. Until the last decade, chiral separations were typically performed with 5 or 3 µm fully porous particle (FPP) packed columns and the times to separate enantiomeric mixtures easily exceeded 30 min, since the analytical throughput of chiral LC–MS is primarily dependent on the efficiency of chiral columns[145]. Since the introduction of short columns, packed with very efficient chiral stationary phases (CSPs), such as sub-2 µm FFPs and sub-3 µm SPPs, operated at high flow rates, the separation of racemates can nowadays be done in very short times (often less than 1 s) in normal-, reversed-phase and HILIC conditions[146]. Chiral LC coupled with tandem MS combines the advantages of the highly selective separation capacity of chiral LC and the superior sensitivity and specificity of MS detection. In this respect, ammonium acetate and ammonium formate are increasingly used for the enantioseparation of basic drugs to improve MS compatibility. Hellinghausen and coworkers used and compared four core-shell CSPs for the enantiomeric LC-ESI(+)-QQQ-MS separation of 150 chiral amines with a variety in structural and drug classes. The CSPs included macrocyclic glycopeptide-based CSPs (VancoShell and NicoShell), cyclodextrin-based CSP (CDShell-RSP) and cyclofructan-based CSP (LarihcShell-P). A resolution between 1.5 and 2.5 was achieved for all 150 enantiomeric amines. Cyclofructan-based CSP performed best for primary amines, while cyclodextrin- and glypopeptide-based CSPs separated a variety of primary, secondary and tertiary amines. The glypopeptide-based CSP showed the best overall performance, and provided the most complementary separation of the pharmaceuticals[147]. An efficient separation of miconazole enantiomers in RPLC mode was achieved on a polysaccharide-based CSP using an ESI(+)-QQQ-MS in MRM mode[148]. Hasan and coworkers developed three different LC-ESI(+)-QQQ-MS assays for the quantitative chiral and achiral determination of ketamine (KET, anesthetic drug) and its metabolites in human serum, urine and faeces. To separate the *S*- and *R*-enantiomers of KET, n-KET and DHNK, a gradient was applied with a mobile phase containing

ammonium acetate and isopropanol on a protein-based CSP, while the enantioselective separation of the HNK metabolites was done on an amylose-based CSP. An ESI-QQQ-MS was coupled to the LC system for the detection[149]. In another contribution, Guo and coworkers used a vancomycin-based (macrocyclic glycopeptide) 100 × 2.1 mm CSP core-shell 2.7 µm column, and compared its performance with a 5 µm FPPs based analogue column. The core-shell column provided higher efficiencies, a 2–5 times greater sensitivity and a shorter analysis time for a set of 22 basic pharmaceutical drugs by using an ESI(+)-QQQ-MS detector[150]. Todoroki et al developed a LC-ESI-QQQ-MS/MS method to detect 11 chiral pharmaceuticals and their hepatic metabolites using a 3 µm, 150 × 2.1 mm ovomucoid chiral column. All the pharmaceuticals examined were enantioseparated with a resolution > 0.82[151].

3.5. Pharmacokinetics/pharmacodynamics (PK/PD) studies

ADME-TOX (Absorption, Distribution, Metabolism, Excretion and Toxicity) studies are performed during the discovery, lead optimization and preclinical development of new drug entities to provide information to characterize these compounds based on their properties and predict their fate in the human body after administration. Compounds with poor ADME properties are preferably removed as early as possible in the discovery phase instead of during more costly drug development phases. The overall process of finding the best candidate for clinical development is long and complicated, requiring numerous studies and a constant redesigning of the chemical entities under development. The strategy for drug discovery and development, including pharmacokinetics and toxicity studies, has drastically changed in the last decade because of the improved understanding of the associated molecular and cellular events, and the development of highly sensitive analytical instruments. LC–MS based techniques are currently the most powerful and reliable analytical techniques in the field of pharmacokinetics/pharmacodynamics (PK/PD) studies including therapeutic drug monitoring (TDM)[152].

In 2017, the tissue distribution and percutaneous absorption of indometachain (IND, non-steroidal anti-inflammatory drug) patches were studied using commercial IND as a comparison. The concentration of IND in skin, plasma, and muscle in mice was measured by UHPLC-ESI(+)-QQQ-MS in MRM mode and the IND concentration in the dermis of rats was also monitored by microdialysis. It was concluded that the examination of the tissue distribution and the application of a microdialysis technique provided an effective means to evaluate indomethacin pharmacokinetics[153]. In the same year, the pharmacokinetic profiles and tissue distribution characteristics of clevidipine (antihypertensive drug) and its primary metabolite H152/81 were investigated by using a RPLC-ESI(+)-QQQ-MS in MRM mode after intravenous administration to rats. The experimental data showed that clevidipine was quickly eliminated from blood with a half-life of about 4.3 min and rapidly distributed in all the investigated tissues after administration. The highest concentration of clevidipine was found in the heart whereas the lowest concentration was detected in the liver. In addition, clevidipine was almost undetectable in most tissues except for heart and brain at 90 min post-dosing, suggesting that there was no apparent long-term accumulation in rat tissues. H152/81 was found at significant concentration levels in all tissues at each time point[154]. In 2018, Kucwaj-Brysz and coworkers, studied the effect of the introduction/deletion and the mutual orientation of aromatic rings in animal models in their search for selective and active serotonin 5-HT7R antagonists among 3,5-disubstituted arylpiperazine-imidazolidine-2,4-diones. To support the structure of synthetized drugs, NMR and LC-ESI-QQQ were employed and *in-vitro* ADME-TOX studies were performed, reporting the low cytotoxicity of the synthetized compounds[155]. Recently, Tanner et al. studied drug-like properties (microsomal/plasma protein binding, *in vitro* microsomal stability, lipophilicity, kinetic solubility and passive permeability) and *in vivo* PK parameters of three decoquinate derivatives (potentially anti-TB

drugs) after oral and intravenous administration to male C57BL/6 mice using a RPLC-ESI-QIT-MS system. The authors claimed that, although all three compounds (RMB_{041}, RMB_{043}, and RMB_{073}) had similar *in vitro* ADME properties, RMB_{041} was the preferred compound to be taken forward in further studies due to its longer *in vivo* half-life and higher bioavailability[156].

4. Conclusions

An overview of the main critical aspects and future trends in LC-MS based techniques in the pharmaceutical field for the analysis of small pharmaceutical molecules is reported. In the last decade, LC-MS based techniques have shown great potential for the analysis of pharmaceuticals, and have played an important role in the study of drug metabolism, the discovery of new drug candidates and the identification and characterization of degradation products and impurities in drug products. Recent innovations in LC instruments, chromatographic modes and stationary phases have turned LC into the golden standard for analytical strategies in the pharmaceutical field. In particular, the development of miniaturized LC systems, such as microfluidic chip-based and more reliable nano-LC systems are attracting attention and can potentially become more important in the future in pharmaceutical analysis. Moreover, due to the commercialization of easy-to-operate and cheaper LRMS (in particular quadrupole MS), and the easier accessibility of more performant MS technologies such as HRMS instruments, MS will most likely expand even more as a preferred detection method for pharmaceutical applications. Even if quadrupole MS technology is still predominant in pharmaceutical analysis, HRMS is becoming not only feasible but also necessary for a confident determination in the pharmaceutical field.

5. References

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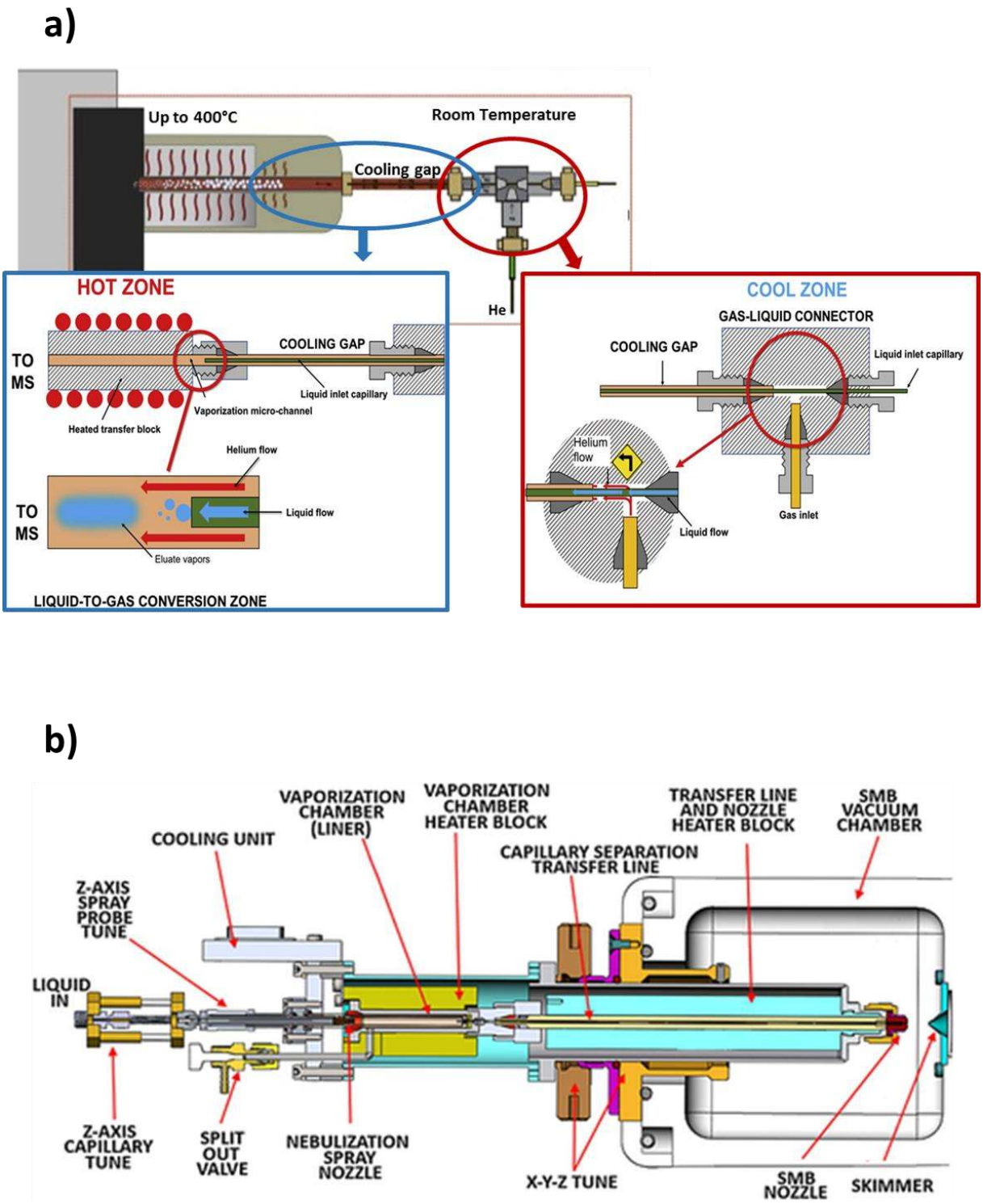

Figure 1: **a)** Detailed scheme of the LEI interface. Reproduced from ref. 31 with permission from Elsevier, copyright 2019. **b)** Scheme of the vaporization chamber in the optimized SMB LC-EI MS system. Reproduced from ref. 33 with permission from John Wiley & Sons, Ltd., copyright 2015.

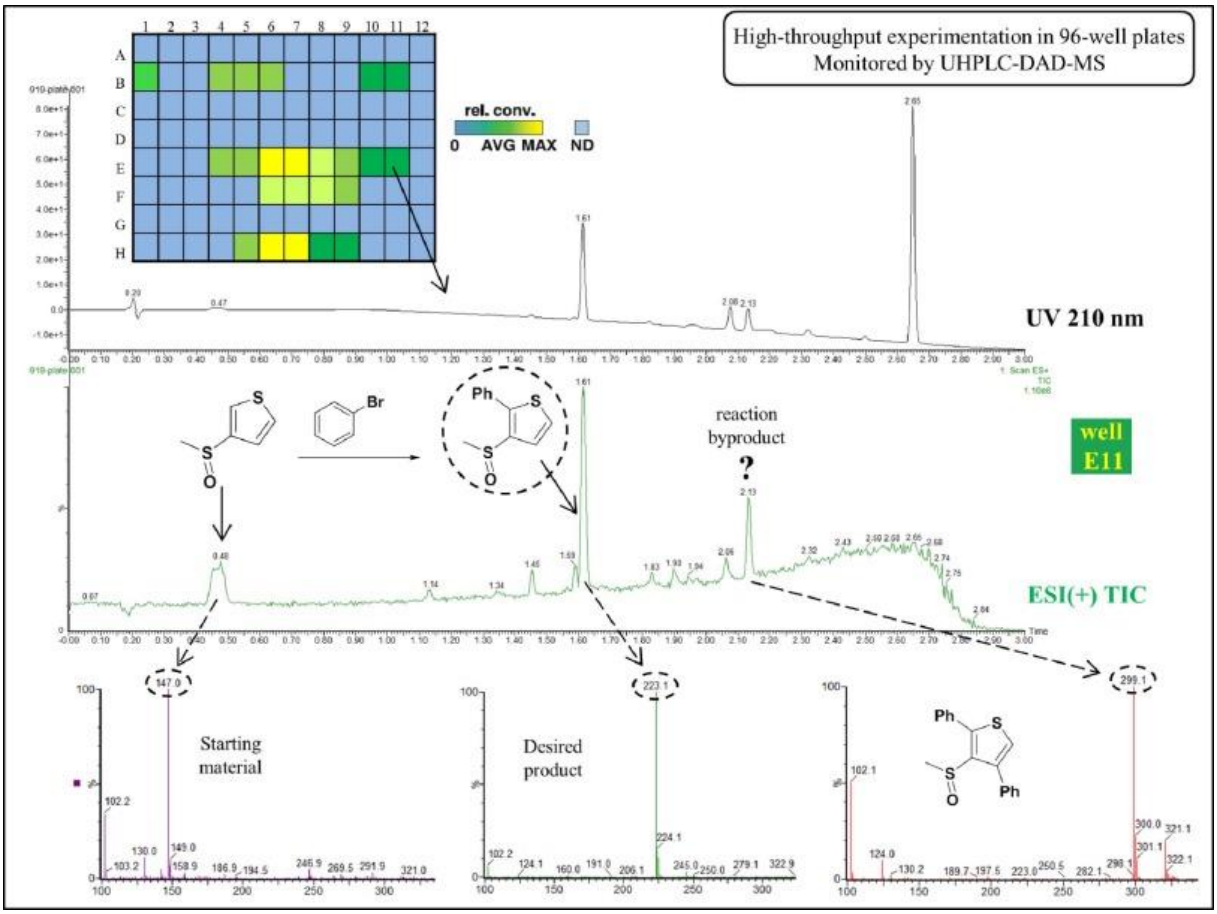

Figure 2: High-throughput analysis of a 96-well plate (highlighting the injection of well E11) monitored by UHPLC-QDa MS. The incomplete conversion of the starting material ($[M+H]^+$= 143 amu) to the desired product (m/z = 223 amu) is shown, while the presence of a reaction byproduct (m/z = 299 amu) is also indicated. Reproduced from ref. 38 with permission from Elsevier, copyright 2016.

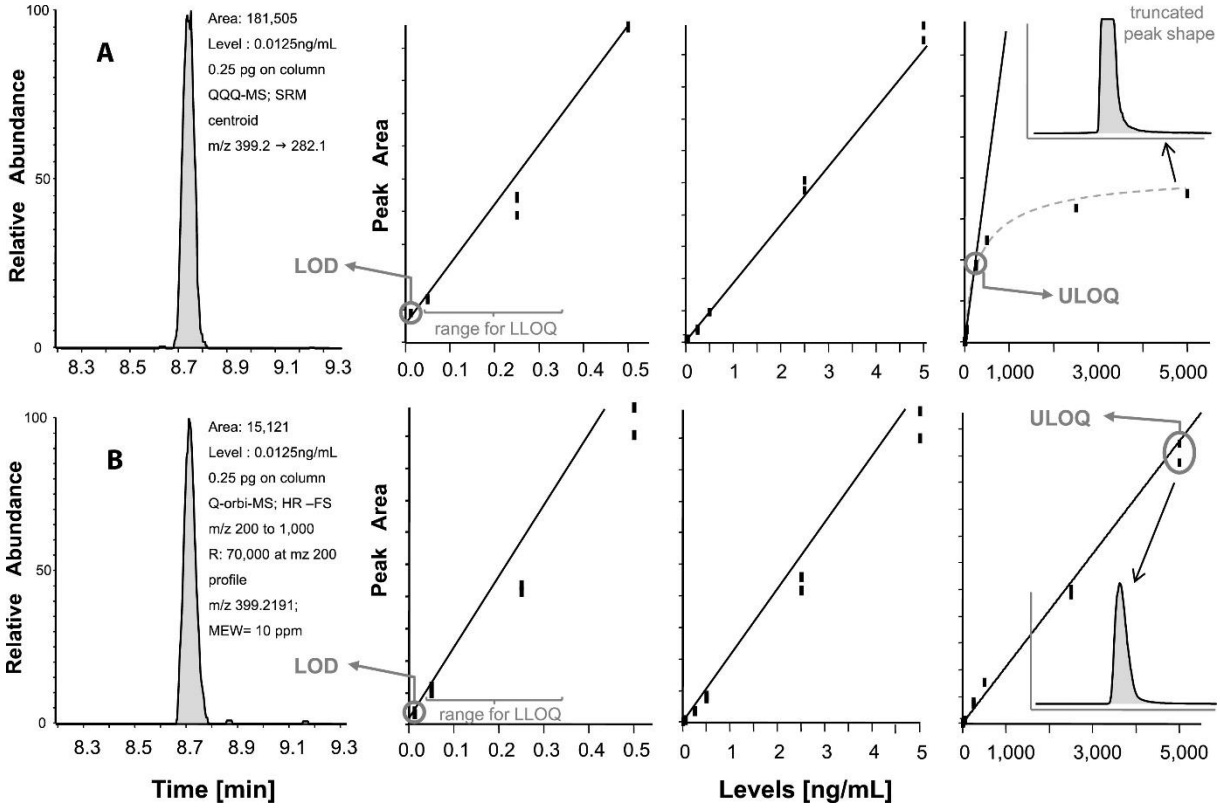

Figure 3: Calibration curves of sunitinib spiked in plasma extracts, from LC-ESI$^+$-MS analyses performed with a QQQ-MS (A) and HRMS (B) acquiring SRM and HR-FS acquisitions, respectively. Chromatograms (on the left-hand side) show sunitinib at the LOD level (0.0125 ng/mL). Truncated peak shape found at high concentrations on the QQQ-MS indicates a saturation of the detector. Reproduced from ref. 43 with permission from Elsevier, copyright 2016.

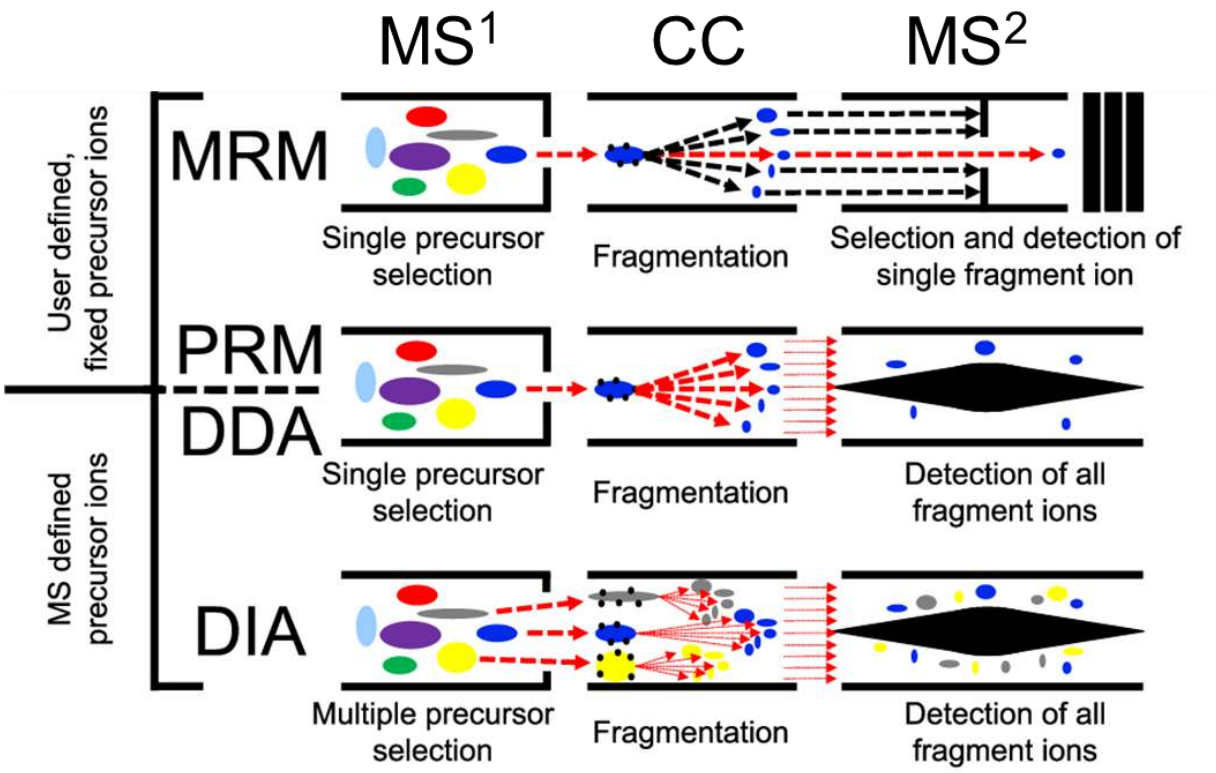

Figure 4: A generic scheme of how analytes are isolated, fragmented and analyzed by a mass spectrometer working in multiple reaction monitoring (MRM), parallel reaction monitoring (PRM), data-dependent acquisition (DDA), or data-independent acquisition (DIA) modes. Reproduced and adapted from ref. 52, F1000research (open access), copyright 2016.

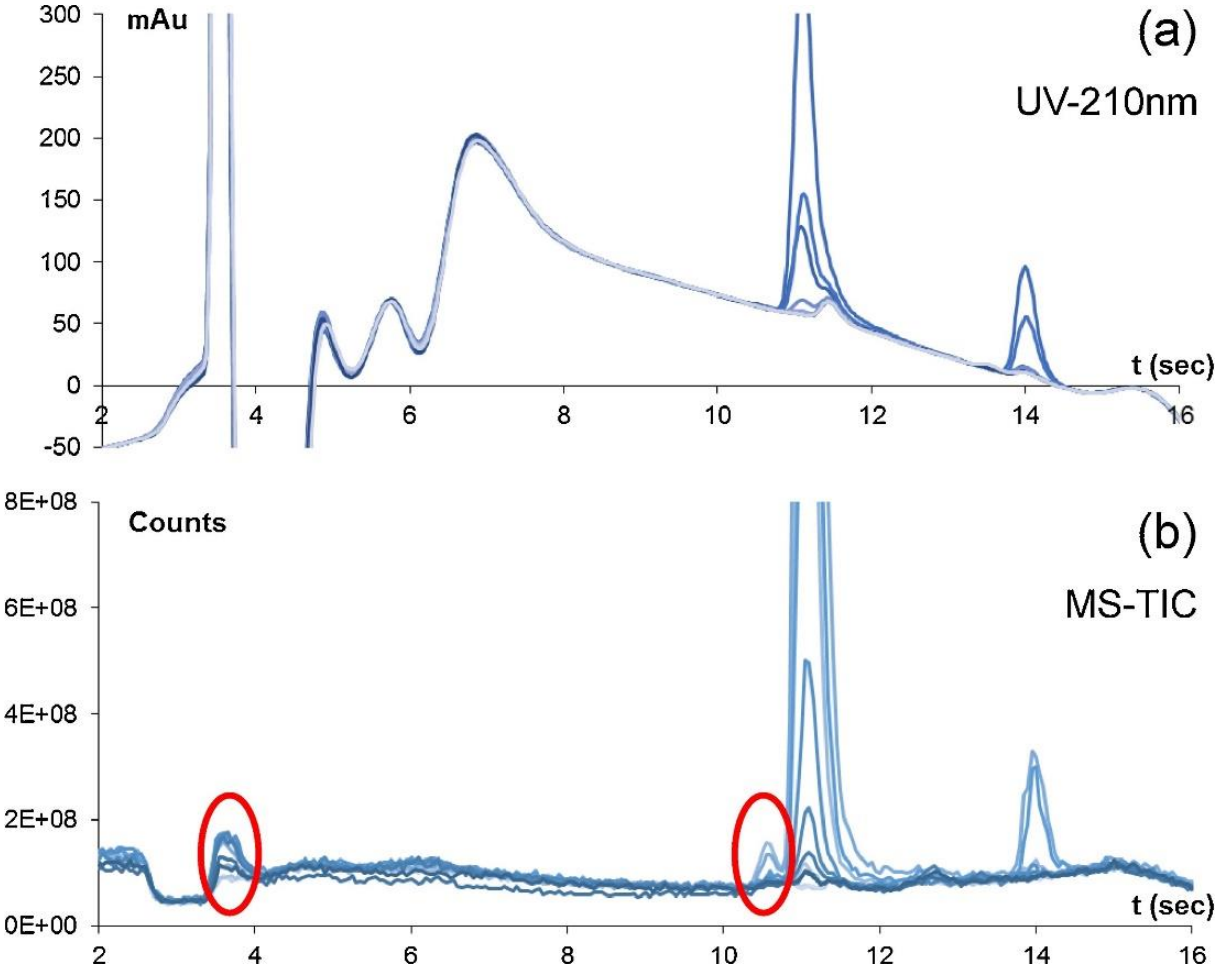

Figure 5. Overlaid 2D separations of 1D fractions, indicated by blue areas. **(a)** UV detection and **(b)** MS-TIC detection signal. Circled compounds are detected in MS but not in UV. Reproduced from ref. 97 with permission from Elsevier, copyright 2017.

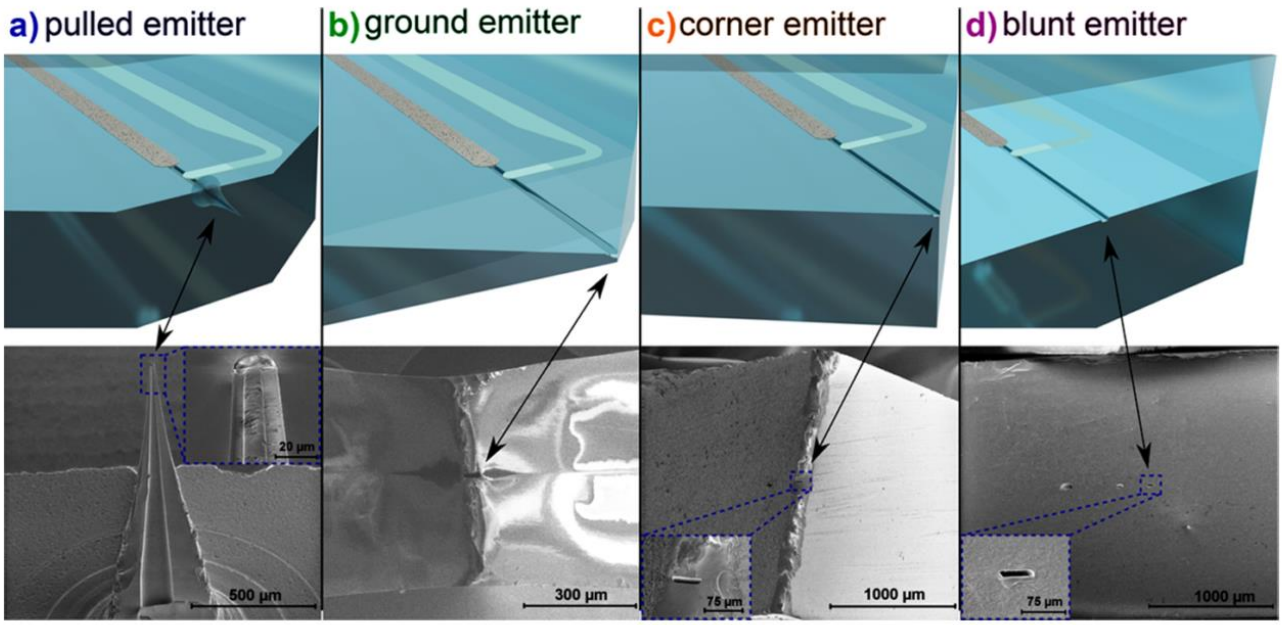

Figure 6. Schematic drawings (top row) and SEM images (bottom row) of different emitter geometries. The elution channel is depicted about three times bigger than in the actual chip. The SEM image of the pulled emitter shows the emitter in a side view with removed cover glass slide, revealing the tapered etched channel. The other geometries are shown in frontal view. Reproduced from ref. 103 with permission from the American Chemical Society, copyright 2016.

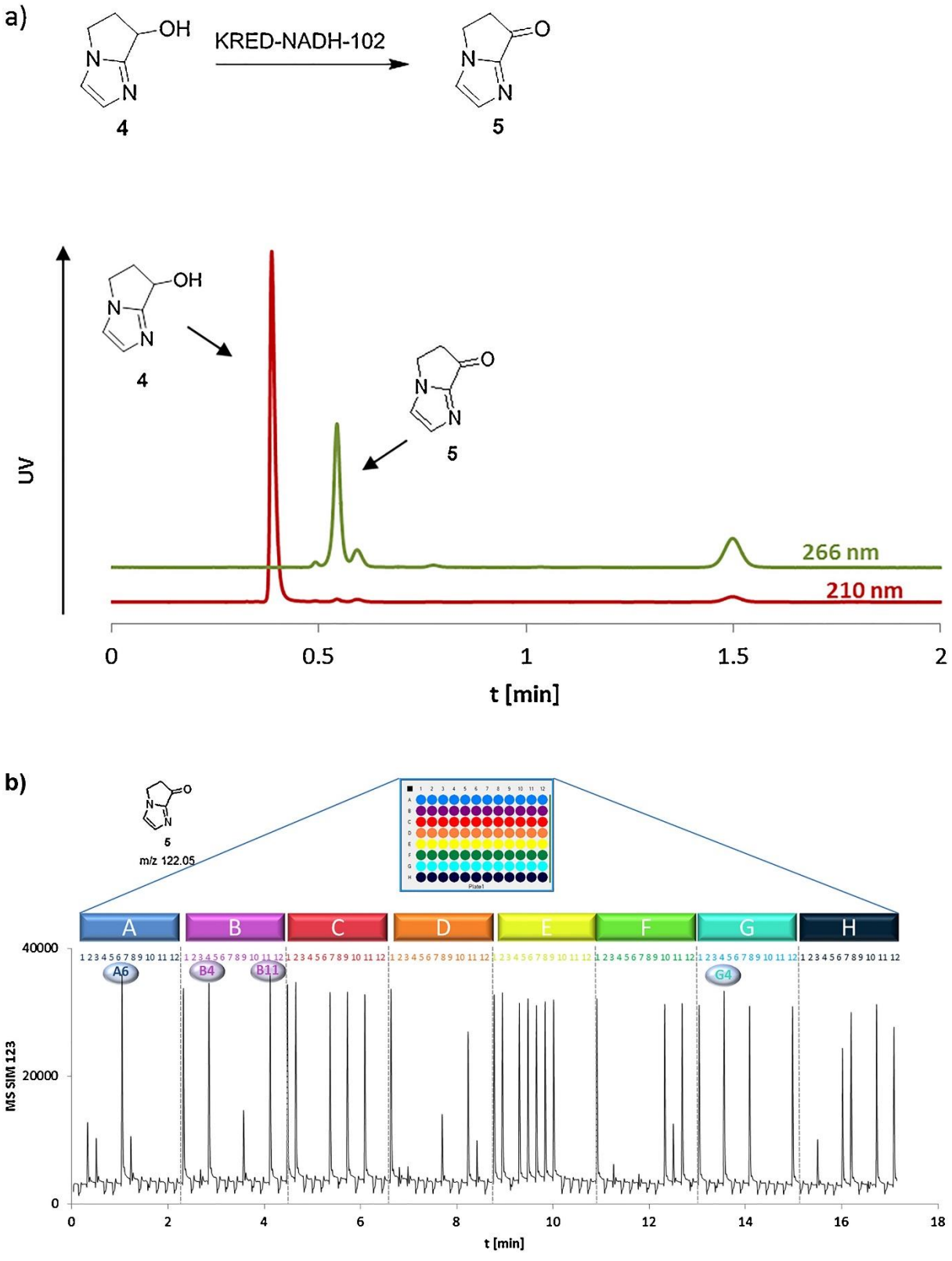

Figure 7. MISER analysis to support high throughput screening of ketoreductase variants produced by site saturation mutagenesis. **a)** Reaction scheme highlighting the desired oxidation of the alcohol (4) to the desired ketone (5). **b)** MISER analysis of product formation enables analysis of 96 reactions in 17 min. Reproduced from ref. 132 with permission from Elsevier, copyright 2017.