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Recent advances in chromatography for pharmaceutical analysis

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¶ V.DA. and S.F. equally contributed to this Review.

1. Introduction

The pharmaceutical industry is one of the most regulated industries worldwide, since the drug products have to be safe and effective. The levels of impurities and degradation products in active pharmaceutical ingredients (APIs) have to be strictly controlled and meet the specifications required by international authorities. Chromatography has been the technique of choice for many years to assess the chemical purity of drug substances and products and is widely used in the pharmaceutical industry, from research and development to quality control (QC) laboratories.

Reversed-phase liquid chromatography (RPLC), which consists of a polar mobile phase and an apolar stationary phase, is the most appropriate technique to analyze mildly polar to apolar substances, that is, substances having an octanol-water partition coefficient (log P) between -1 and 5.[1] Currently, RPLC is considered to be the gold standard in pharmaceutical analysis, and its success is attributed to the fact that this chromatographic mode matches perfectly with the physicochemical properties of drugs. Indeed, Lipinski developed his famous rule of thumb approximately 20 years ago to evaluate the drug-likeness of chemical compounds. Among the different criteria, he stated that an orally active drug should have a log P not greater than 5.[2] A few years later, this rule spawned some extensions, and it was demonstrated that log P should, ideally, be between -0.4 and 5.6.[3] Considering this log P range, it is clear that drug lipophilicity fits well within the RPLC retention window.

In addition to lipophilicity, another important characteristic of drugs is their ionic properties, and a significant proportion of drugs are ionizable (approximately 65% according to the world drug index),[4] including a large majority of basic substances (75% of the ionizable drugs). In RPLC, it is well known that stationary phases should be deactivated (i.e., decrease silanol activity) to properly analyze basic drugs, as these drugs can interact with negatively charged residual silanols, leading to severe peak broadening and tailing. In the 1990s and later, many innovative strategies were developed by column providers to analyze basic drugs, including innovative end-capping procedures, ultra-pure silica, development of hybrid silica materials and high-pH stable stationary phases. Consequently, most of the innovations in column technology were implemented to answer the needs of the pharmaceutical industry.

Due to the increasing costs and complexity of the drug development process in the early 2000s, there have been requests from the pharmaceutical industry to increase the overall performance of LC in terms of throughput and resolution. To achieve this goal, ultrahigh performance/pressure liquid chromatography (UHPLC) was developed in 2004 and consisted of using columns packed with sub-2 µm particles in combination with a chromatographic instrument capable of withstanding pressures up to 1000 (which has been recently extended

up to 1500) bar.[5] Using this strategy, analysis times of 1 - 4 min (a valuable approach for fast method development)[6] and/or a peak capacity of 300-700 (required for complex impurity profiling) could be easily achieved.[7]

Since 2011, there has been a growing interest in protein biopharmaceuticals (particularly monoclonal antibodies and related compounds) as new therapeutic agents in the pharmaceutical industry. Due to the specific analytical needs of these novel classes of biomolecules, several innovative chromatographic columns were introduced into the market. These columns possess i) large pore sizes that limit restriction to diffusion and exclusion (300-1000 Å), ii) enhanced kinetic performances to elute large solutes in sharp peaks (use of superficially porous particles)[8,9] and iii) improved chemical inertness,[10] to limit protein adsorption and reduced silanol activity. In addition to the chromatographic stationary phases, bioinert LC systems (mostly plumbed with titanium) also appeared on the market.

Today, all of the above mentioned chromatographic innovations (i.e., deactivated stationary phases, UHPLC technology, columns/systems dedicated to biopharmaceutical analysis) have been integrated into most pharmaceutical laboratories involved in research and development. This allows for successful and rapid development of methods for the analysis of various APIs and products. However, this is still not the case in QC laboratories, since the employed methods have to be validated, which is why many of them are still running old-fashioned methods involving diethylamine, as a mobile phase additive to limit silanol activity, in combination with older-generation stationary phases 250 mm in length and packed with ≥5 µm particles.

The goal of this contribution is to review the current and future trends in chromatography (mostly LC) applied to pharmaceutical analysis. Among the most important trends of the last few years, high-throughput analysis and automation, 2D-LC, supercritical fluid chromatography (SFC), hydrophilic interaction chromatography (HILIC), the combination of LC with low-cost MS detectors, automated method-development software, green LC and the latest advances in GC are discussed in this article. In addition to these technological advances, several specific pharmaceutical applications are also illustrated, including the analysis of protein biopharmaceuticals, chiral drugs, genotoxic impurities, process analytical technology (PAT), and cleaning validation.

2. Possibilities in high-throughput and high-resolution LC separations

There has been a huge interest in UHPLC and narrow-bore columns packed with sub-2 µm fully porous particles (FPPs) since its commercial introduction in 2004, which dramatically increased the throughput of conventional HPLC methods.[5] This high-throughput feature is

attractive for environmental, food, and chemical analyses, where productivity needs to be increased due to the large number of samples. However, the main driving force for developing fast separations is the pharmaceutical industry. Indeed, enhanced productivity and reduced costs are particularly needed during the drug discovery and development processes in applications such as quality control, pharmacokinetics and drug metabolism. Typical pharmaceutical separations can be performed in 2 – 10 min intervals with current UHPLC technology, and complete method development can be carried out in 1 – 2 days. However, it is important to keep in mind that detectors, connection tubing, gradient mixers and mobile phase preheaters had to be redesigned to be compatible with the very narrow peaks obtained by these very efficient small columns. When working with UHPLC columns of 50 × 2.1 mm packed with sub-2 µm particles instead of regular HPLC columns of 150 × 4.6 mm packed with 5 µm particles, the analysis time can be decreased by a factor of 5 – 9, while maintaining an equivalent kinetic performance.[5]

In addition to FPPs, superficially porous particle (SPP, also known as fused-core or core-shell) technology has also received considerable attention over the past few years.[8] In practice, SPPs combine the benefits of both FPPs and nonporous particles. The high efficiency achieved by columns packed with sub-3 µm SPPs, combined with convenient operating conditions (modest back pressures and the ability to use conventional HPLC instruments), has enabled the success of current SPPs.[11] Columns packed with sub-3 µm SPPs rival the efficiency of columns packed with sub-2 µm FPPs for small drugs, but the former generate only half the backpressure of the latter. As a result, practitioners can use SPP columns on regular HPLC equipment. Moreover, further performance improvements have been achieved by using very fine SPPs (1.3 – 1.7 µm), and through the use of ultralow dispersion UHPLC systems.[12] Today, SPPs are routinely applied to pharmaceutical and biopharmaceutical analyses. The morphology of SPPs is especially beneficial in the analysis of slowly diffusing compounds (large proteins) as the mass transfer kinetics become faster, since the solute diffusion path is decreased. For this reason, several wide-pore SPPs with nominal pore sizes between 200 and 1000 Å are now available from various vendors. Recently, so-called radially oriented mesoporous (ROM) SPPs were proposed to further decrease the longitudinal diffusion and hence improve the overall separation efficiency.[13]

A possible solution to further improve the separation throughput is to extend the pressure limits of current UHPLC systems, as demonstrated by Jorgenson and Lee some years ago, by using capillary columns with an I.D. of 30 - 50 µm. More recently, it was shown that the pressure limits for narrow-bore columns (2.1 mm ID) could be increased beyond the limits of commercially available instrumentation. A prototype LC setup was realized, allowing operation at pressures up to 2600 bar.[14] The performance of coupled columns packed with

FPPs and SPPs was assessed under ultrahigh-pressure conditions. A maximum plate number of 81,000 was realized using a 600 mm long column at 2600 bar. However, working at elevated pressures and high flow rates requires consideration of the frictional heating that results from pumping the mobile phase through the column. This so-called viscous heating, or viscous dissipation of the mechanical energy, increases the temperature of the mobile phase, column bed and hardware (i.e., wall, fittings, frits). Depending on the oven design (i.e., still- or forced-air), the development of axial and radial temperature gradients is expected, which can significantly impact solute retention and band broadening, respectively.[15] In addition to the reduction of column diameter, a possible solution to eliminate detrimental heat effects is to insulate the coupled columns and apply an intermediate-cooling strategy in a forced-air oven. Recently, experimental measurements confirmed that an ~60 °C relative temperature increase occurs when operating a 100 x 2.1 mm column at 2600 bar using methanol as the mobile phase.[16]

Another possibility to improve separation times is to work with very short columns (≤ 1 cm) packed with very efficient particles and run steep gradients. However, the handling of poorly retained and very narrow peaks in the domain of conventional HPLC/UHPLC is currently hindered by extra-column dispersion and data sampling rates. Obtaining ideal chromatographic outputs from very efficient columns and fast eluting peaks is hindered by several factors. The shape of the injector pulse, the flow distribution pattern of the inlet and outlet frits, diffusion and mixing in plumbing unions, flow profiles in the connector tubing and the data sampling rate, all affect the true peak shape and width in deleterious ways.[17] Despite these physical limitations, a recent study demonstrated the potential of performing subsecond separations using guard columns, which were 5 mm long standard bore columns packed with 2.7 µm SPPs, on a modified commercial UHPLC system.[18] To operate the UHPLC instrument at the highest flow rate possible (5.0 mL/min), the in-line filter was removed. Several modifications were made to a commercial LC system to decrease the extra-column volume and band broadening. The raw chromatographic signals were further processed by deconvolution and peak fitting and finally transformed with a power function. This setup enabled the separation of 2 – 3 compounds in the second range, operating the 5 mm columns at a rate of 5 mL/min on the optimized system. Figure 1 shows an example of a 1.2 sec separation of 3 acidic compounds.

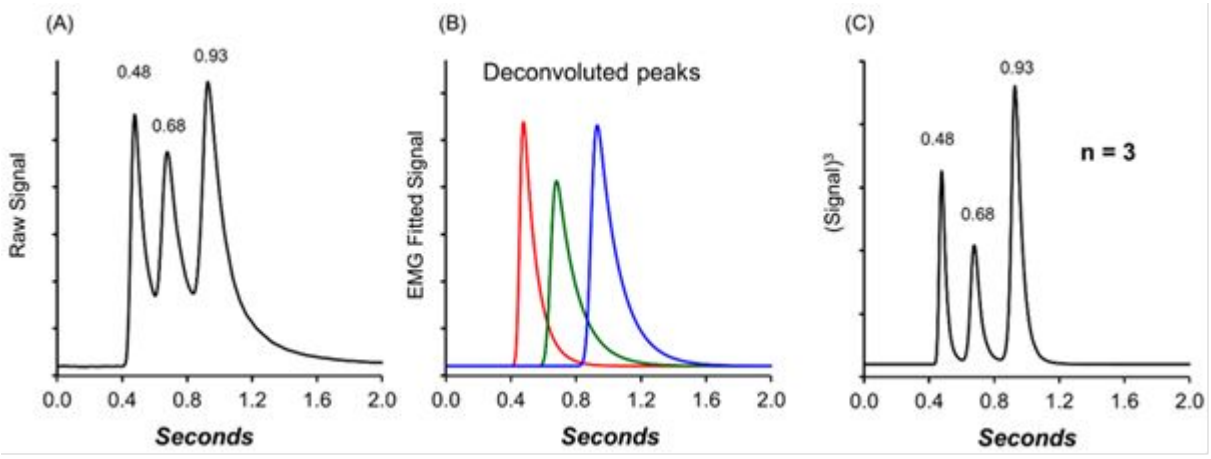

Figure 1. Application of a power transformation to the subsecond chromatography of three components (mellitic acid, 2,3-dihydroxybenzoic acid, and 4-aminosalicylic acid). (A) The original subsecond chromatogram. (B) Deconvolution of the chromatogram into three exponentially modified Gaussian peaks. (C) Power transformation with the cube of the original data. Reproduced from Wahab, M. F.; Wimalasinghe, R. M.; Wang, Y.; Barhate, C. L.; Patel, D. C.; Armstrong, D. W. Anal. Chem. 2016, 88 (17), 8821–8826 (ref [18]). Copyright 2016 American Chemical Society.

It has been shown several times during the past few years that the time-limiting steps of high-throughput UHPLC analysis (shorter than 1 min) are the injection cycle and the unused time during the real separation (such as the column void, gradient delay, column equilibration, software setup and offset times). The total time required to perform a separation is often referred to as the "duty cycle" of the LC analysis.[19] A possible solution to decrease the undesired cycle times of consecutive chromatographic runs is to perform simultaneous (parallel) analyses on several columns. This approach is generally called staggered chromatography, but the terms "multiplexed" or "MISER" (Multiple Injections in a Single Experimental Run) are also often used. In such an approach, two to four columns are independently used with the same number of dedicated pumps, and one or two autosamplers inject the samples sequentially onto these columns. Then, a stream selection valve sequentially directs the effluent from each column to either the waste or detector (often MS), depending on the retention times of the analytes of interest. In a four-column setup, only ~ 1/4 of the LC run time is monitored by the detector for each column, while the eluent is directed to the waste the rest of the time. Therefore, the staggered approach is most useful for targeted analysis. Today, several vendors offer staggered systems, such as Transcend II (Thermo), MPX (Sciex) and StreamSelect (Agilent). Recently, a dual needle autosampler was developed and shown to increase the throughput of MISER analysis.[20] This autosampler can be installed into any standard HPLC system and enables a 10 s injection cycle time. Complete analysis of 96 microplates was performed in 17 min.

When the separation is not sufficient on a single column, the best solution consists of increasing the column length by coupling several columns in series. However, as discussed

earlier, throughput speed inevitably decreases due to i) the limitations of the operating pressures of standard LC instruments and ii) the increase of undesired friction and heat effects at very high pressures. An alternative solution consists of using the “recycling LC” concept, where the sample peaks are simply recycled back into the column to continue the separation process until the peaks are separated. For such an approach, either a direct-pumping (using only one column) or an alternating-pumping (using two twin columns and a switching valve) recycling system can be used. The maximum allowable system pressure is no longer a problem, since only two columns are connected in series. Figure 2A shows a schematic view of a two column system, also known as the “twin-column recycling separation process”[21]. Recycled separations enable chromatographers to solve very challenging separation problems caused by the structural similarity of sample compounds. Recycling chromatography has been successfully applied to separate isotopes, isomers and optically active compounds. Figure 2B shows an example of isotope separation using 1 to 22 cycles. Even if this concept is very powerful in theory, the practical performance of the real process can significantly deviate from that of the ideal process, in which the overall resolution factors are directly predicted from the number of cycles and the intrinsic efficiency of the single column. These deviations originate from *(i)* the extra-column band broadening of the sample during sample transfer steps (first column to valve, valve, valve to inline detector, in-line detector to valve, valve, and valve to second column), *(ii)* the potential decrease in column performance due to repetitive valve actuation (and the subsequent transient pressures), which may affect the stability and structure of the packed bed (the two columns switch from high to low and from low to high pressures) and, therefore, *(iii)* the pressure dependence of the solute retention along the column. For these last two reasons, it is preferable to keep pressure drops moderate to reduce the risks of column failure and pressure-dependent retention behavior, which both compromise the performance of the recycling process.[22]

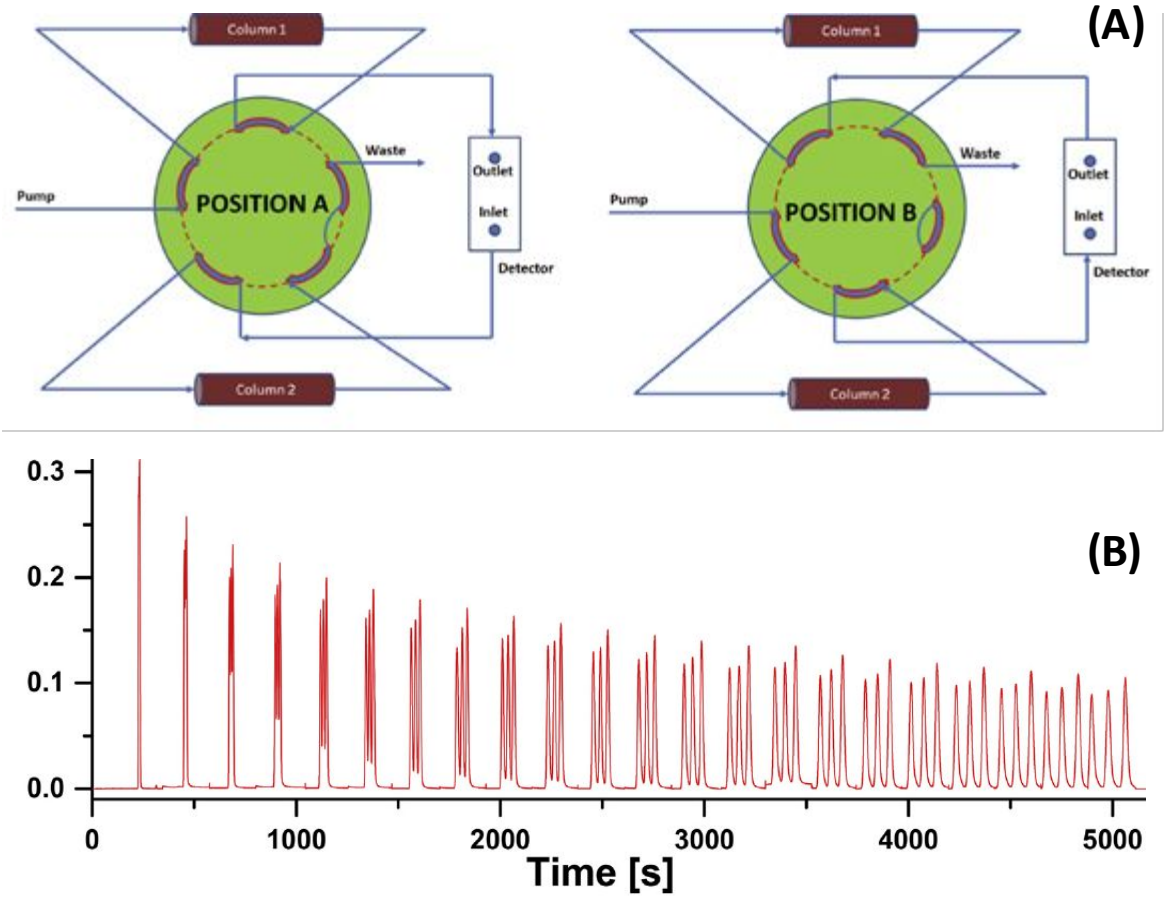

Figure 2. Schematic representation of a twin-column recycling separation system (A) and chromatograms obtained with n=1 to n=22 recycling cycles for a benzene isotope mixture (B). Reprinted from J. Chromatogr. A, Vol. 1524, Gritti, F.; Besner, S.; Cormier, S.; Gilar, M. Applications of high-resolution recycling liquid chromatography: From small to large molecules, pp 108−120 (ref [21]). Copyright 2017, with permission from Elsevier, and Reprinted from J. Chromatogr. A, Vol. 1532, Gritti, F.; Cormier, S. Performance optimization of ultra-high-resolution recycling liquid chromatography, pp 74−88 (ref [23]). Copyright 2018, with permission from Elsevier.

3. Applicability of 2D-LC

2D-LC has emerged as a powerful technique to answer the need for increasing peak capacity to resolve complex sample mixtures (containing either too many or closely related compounds). In fact, the number of peaks that can be individually separated within a given time window strongly depends on the number of compounds present in the sample and the effective peak capacity of the LC system. Stoll and Carr[24] perfectly demonstrated this concept in Figure 3 by showing that, for a mixture consisting of 50 compounds, with an effective peak capacity of 100 (achievable by 1D-LC), only 50% of the components would be resolved as single chromatographic peaks, whereas an effective peak capacity of 3000 would be required to resolve 95% of the compounds. However, such a high peak capacity is only achievable by 2D-LC. This unique ability of 2D-LC is due to the combining of two different liquid separation modes that offer different selectivities (orthogonal separation mechanisms), thus enabling an enhancement of the resolving power.

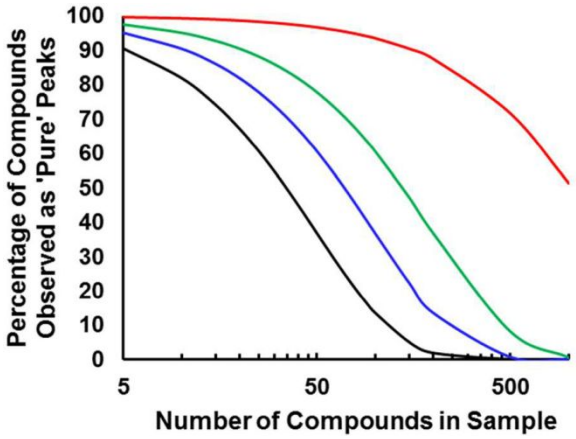

Figure 3. Percentage of compounds that are resolved as a single chromatographic peak (minimum resolution of 1.0) as a function of the number of compounds in the sample and with an effective peak capacity of 100 (black), 200 (blue), 400 (green) and 3000 (red). This behavior is only true when having a random peak distribution over the entire retention window. Reproduced from D.R. Stoll, P. W. Carr, Two-Dimensional Liquid Chromatography: A State of the Art Tutorial, Anal. Chem. 2017, 89, 519-531 (ref [24]). Copyright 2017 American Chemical Society.

Depending on the analytical question to be solved, the choice of the two separation dimensions can be critical, and various specific requirements need to be considered. Among them, we have to mention the complementarity of the stationary phase selectivities, the miscibility of the mobile phases in the first and second dimensions, the impact of the mobile phase nature on injection into the second dimension and the possibility of coupling to MS detection. Additionally, the second dimension separation needs to be very fast (less than 2 min). To date, several combinations of retention mechanisms (selectivities) have been explored, and a comprehensive overview has recently been provided by Pirok *et al.* (Figure 4).[25] In this table, the authors listed the pros and cons arising from the combination of the most common forms of LC separation by evaluating potential peak capacity, column re-equilibration time, and various abovementioned critical requirements, including the degree of orthogonality and the solvent and MS compatibility. At first glance, RPLC is highlighted as the most suitable second dimension mode due to its high robustness, versatility, and applicability, as well as its MS compatibility. In line with this, 2D-LC consisting of two RPLC dimensions has been recently evaluated as the most attractive choice, among 190 combinations of chromatographic systems, for the analysis of pharmaceutical samples in the early stages of drug development.[26] In addition to RPLC, SFC is also a potentially attractive technique as a fast second dimension (Figure 4). In this context, several 2D-LCxSFC applications have recently been proposed for simultaneous achiral-chiral analysis,[27] and the *in vivo* assessment of the ratio of the active pharmaceutical ingredient (API) to its metabolite, as well as evaluating the corresponding enantiomeric excess of each.[28]

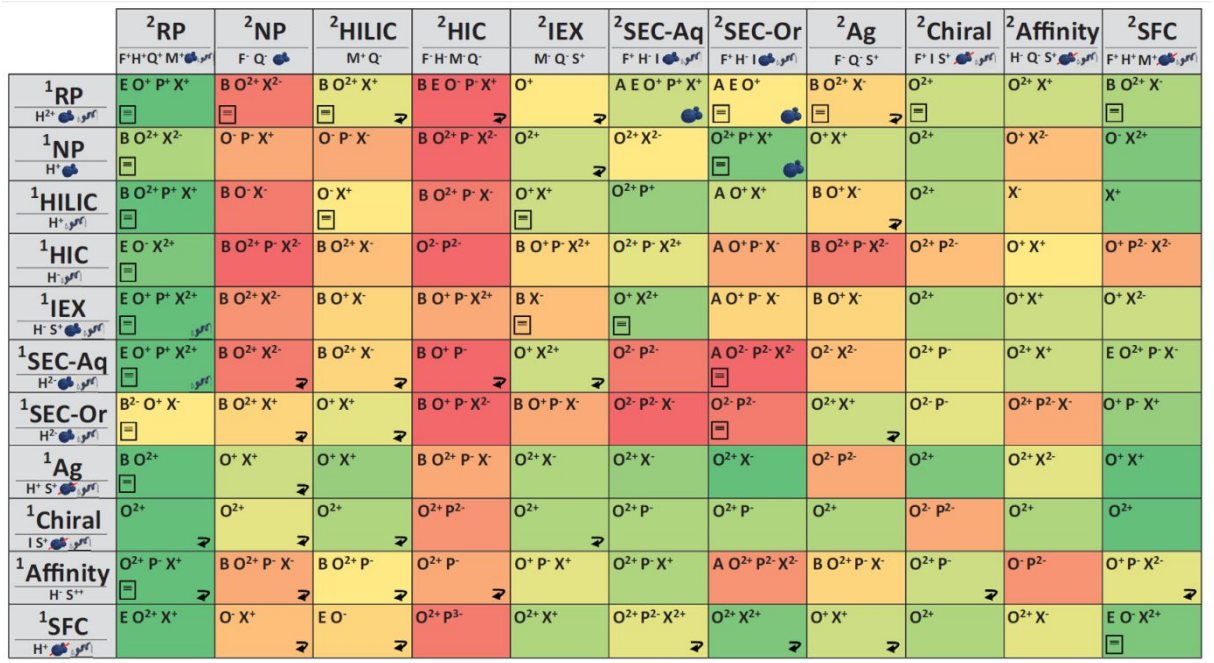

Figure 4. Overview of the possible in-line LC x LC combinations using the most common forms of LC separations. Reprinted from B. W. J. Pirok, A. F. G. Gargano, P. J. Schoenmakers, Optimizing separations in in-line comprehensive two-dimensional liquid chromatography, J. Sep Sci, 2018, 41, pp 68-98. Copyright 2018, with permission from Wiley.[25]

It is clear that the greatest challenge in 2D-LC method development is to overcome incompatibility between the solvent systems, namely, the influence that the effluent of the first dimension can have on the performance of the second dimension. However, Stoll *et al.*[29] paved the way to new possibilities and applications by proposing a valve-based approach, referred to as *Active Solvent Modulation* (ASM), specifically designed to address this incompatibility issue. Without the need for additional instrument hardware, ASM enables the dilution of the effluent of the first dimension with a weak solvent prior to transferring to the second dimension by using an 8 – port / 4 – position valve equipped with a bypass path. The benefits of the approach were demonstrated using small molecule probes and the degradants of heat-treated bovine insulin as case studies.[29]

It is also worth mentioning that several possible implementations of 2D-LC rely on how the effluent of the first dimension is transferred to the second dimension (Figure 5).

Historically, heart-cutting (LC-LC, Figure 5A) was the earliest developed 2D-LC approach, consisting of collecting the peak of interest (single fraction), which was then injected and analyzed in the second dimension. This approach has been widely applied for resolving closely related compounds (i.e., structurally related impurities, isomers or degradation products) in pharmaceutical analyses, [30] especially those performed through the coupling of orthogonal RP selectivities or those involving achiral-chiral analysis.[31]

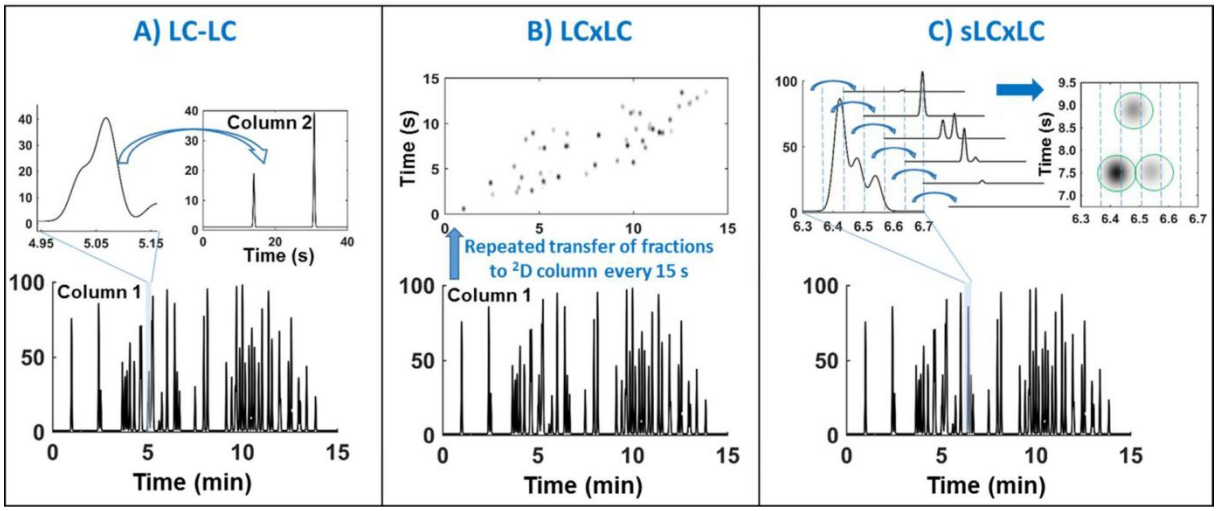

Figure 5. Visual comparison of different implementations of 2D-LC. A) LC-LC (heart-cutting 2D-LC): a single fraction is injected into the 2D column. In multiple heart-cutting (mLC-LC, not shown), more than one region of the 1D effluent would be injected onto the 2D column. B) LC × LC (comprehensive 2D-LC): the entire 1D effluent is sequentially injected. C) sLC × LC (selective comprehensive 2D-LC): segments of the 1D effluent are injected comprehensively onto the 2D column. Reproduced from D.R. Stoll, P. W. Carr, Two-Dimensional Liquid Chromatography: A State of the Art Tutorial, Anal. Chem. 2017, 89, 519-531 (ref [24]). Copyright 2017 American Chemical Society.

Examples include the heartcutting 2D LC-SFC separations of pharmaceutical compounds reported by Venkatramani *et al.*[27] that were enabled through the use of small volume trapping columns to transfer fractions from the first dimension (RPLC, providing the achiral purity result) to the second dimension (SFC, providing the chiral purity result). In another contribution, Iguiniz *et al.* demonstrated the power of 2D LC-SFC separations that included an achiral RPLC column in the first dimension and chiral SFC conditions in the second dimension [REF].[32] Dai *et al.*[33] applied heart-cutting 2D RPLC-RPLC to perform comparison studies of parenteral microdoses of polyethylene glycol formulations in medical devices. Due to the increased selectivity and sensitivity provided by the second dimension, trace impurities were identified and then structurally characterized by MS. Similarly, Yang *et al.*[34] performed heart-cutting RPLC-RPLC to gain advantages from the different column selectivities (C18 *versus* trifunctionally bonded C6 phenyl-ligand stationary phases) involved in the 2D setup. In this study, a coeluting impurity of a pharmaceutical material that was analyzed in the first dimension was resolved from the main peak and analyzed in the second dimension. Interestingly, the authors claimed the suitability of this 2D-LC method for use in good manufacturing practice (GMP) environments. For this purpose, they demonstrated the linearity, accuracy, precision, robustness and sensitivity of the method and validated the suitability and transferability of the 2D-LC setup for quality control (QC) testing. Another heart-cutting achiral-chiral LC-LC method, involving RPLC (C8 phase) in the first dimension and a chiral column (alpha-1-acid glycoprotein) in the second dimension, was used for the successful separation of ketoprofen enantiomers.[35] The experimental setup included factorial

designs as a mathematical approach to allow for the easy optimization of both separation conditions, with a focus on the compatibility of the mobile phases.

Beyond the coupling of orthogonal RP selectivities, heart-cutting 2D-LC was also applied to other LC modes. For example, Wang *et al.*[36] proposed the characterization and quantification of polar excipients in protein formulation samples by heart-cutting SEC-HILIC 2D-LC. Namely, histidine degradation was tracked in complex sample matrices, and its main degradant was identified as trans-urocanic acid. In addition, quantification of the degradant was achieved thanks to the combining of the 2D-LC setup with stable-isotope labeling MS. In a different setup, Luo *et al.*[37] used RPLC as the second dimension in a heart-cutting 2D-LC MS system to perform an in-line desalting step prior to MS detection. Separation of the compounds of interest (a synthetic peptide containing thirty amino acid residues) was performed exclusively by the first RPLC dimension using an $NaClO_4$ additive in the mobile phases. $NaClO_4$, which is an MS-incompatible ion-pairing agent, was then separated in the second dimension and discarded prior to the entry of the effluent of the second dimension into the MS. Several examples were provided to validate the versatility of the approach, and the separation of complex mixtures of peptides containing impurities and positional isomers was extensively discussed.

Compared to the heart-cutting 2D-LC mode, the multiple heart-cutting approach (mLC-LC) allows for more than one region of the first dimension to be injected into the second dimension. An innovative application of mLC-LC for the analysis of pharmaceutical impurities was proposed by Zhang and coworkers, who used a primary column in the first dimension and six orthogonal columns in the second dimension to enhance the flexibility and selectivity. In this configuration, heart cuts from the first dimension were directed into “parking” loops, where they remained until being injected into the second dimension. The tested approaches involved an mRPLC-RPLC setup, including phenyl and C18 as the stationary phases, and were able to reveal hidden degradation products that coeluted with the main drug peak. In addition, an mRPLC-HILIC setup was able to resolve polar component that was poorly retained in the first dimension. Through mLC-LC consisting of SEC and ion-pairing RPLC, Ouyang *et al.*[38] were able to identify and assign more than 80 and 120 oligosaccharides, respectively, from the low molecular weight heparins (LMWHs) nadroparin and enoxaparin. In particular, the 2D-LC approach was emphasized as being of the utmost importance for profiling analysis, where LMW components of either the same or different sizes, but having different charges and polarities, are resolved in the same analysis.

In contrast to LC-LC and mLC-LC, the comprehensive 2D-LC approach (LC × LC, Figure 5B) allows the entire effluent of the first dimension to be injected onto the second dimension. This approach is often used for the separation of very complex samples (especially in *–omics*

applications) or, more generally, when the goal is to gain as much information as possible from the samples. Sarrut and coworkers[39] highlighted the pros and cons of this approach by comparing the results of one-dimensional (1D-RPLC) *versus* in-line comprehensive two-dimensional LC (RPLC × RPLC) for optimized, sub-hour separations of complex peptide samples. It was shown that, within an analysis time of 60 min, the same peak intensity was observed with both techniques but a 3-fold lower injected volume was used in RPLC × RPLC. In addition, coupling to an MS was more advantageous when performing RPLC × RPLC based on a significant increase in the signal-to-noise ratio (strong noise reduction), which suggests that RPLC × RPLC–MS is a promising technique for peptide identification in complex matrices. Stoll and coworkers[40] evaluated the impact of comprehensive LC × LC on UV detection sensitivity during the analysis of degraded APIs. A systematic evaluation of the impact of the volume and solvent composition of fractions of the first dimension on the ability of the second dimension to resolve and detect low-abundance compounds was performed. Interestingly, it was found that dilution of the first dimension effluent with a weak solvent prior to injection into the second dimension had the beneficial effect of concentrating the analyte band in the second column, causing an overall improvement in the sensitivity. Corgier *et al.*[41] used LC × LC for another interesting application by a proof-of-concept investigation on the potential of the technique for micropreparative separations of simple samples. In this work, the authors[41] investigated the usefulness of the approach for recovering minor components from a simple mixture that can be separated by 1D-LC. A test sample of four compounds was separated by two 1D-LC methods and one LC × LC method. A comparison of the preparative performance of the three methods showed that in-line LC × LC was able to achieve a 12-fold increase in the amount of compounds recovered per injection compared to 1D-LC. At the same time, the production rate was increased by a factor of 9.5.

Finally, the selective comprehensive 2D-LC approach (sLC × LC, Figure 5C) allows segments of the first dimension to be comprehensively injected into the second dimension, with the advantage of having the peak volume of the first dimension divided into smaller volume fractions that have less of an impact on the second dimension separation. As reported by Stoll and coworkers,[30] sLC × LC is generally applied to the determination of peak purity during method validation as an effective alternative to UV-vis, diode-array, or MS detection. As an example of this application, Venkatramani *et al.*[42] demonstrated the ability of sLC × LC to resolve structurally similar impurities that eluted in proximity to the main component, with coeluting impurities resolved and detected at levels <0.05% in the presence of the main component. Similarly, Shackman *et al.*[43] highlighted the suitability of the sLC × LC for the determination of peak purity of a pharmaceutical formulation containing multiple active components that possessed diverse physicochemical properties. The analytical

platform allowed for drug product impurity and degradant profiling in the first dimension (a total of 14 targeted fractions were sampled across the first dimension main peak), and the assay of the fractions through a drug substance profiling method in the second dimension. This degree of sampling allowed for the profiling of a coeluting degradant present at a level of 0.2% w/w throughout the main peak.

It is clear that 2D-LC is attracting significant increasing interest and that the inclusion of this technique in the analytical toolbox of pharmaceutical analysis can go beyond increasing the peak capacity, as recently discussed by Haidar Ahmad *et al.*[44] by highlighting three different applications of 2D-LC in the pharmaceutical industry. First, 2D-LC was applied to match the retention times for peaks of interest under different mobile phase conditions by "transforming" an incompatible MS buffer into a MS-compatible buffer. Collection of the peaks of interest was performed in the first dimension, via a heart-cutting method, and injected into the second dimension, where a volatile LC-MS-compatible buffer was used. The second application involved the development of a 2D-LC method able to simultaneously detect and quantify degradation products in a pharmaceutical material that contained both small and large molecules. Finally, the third application had the goal of using 2D-LC to support stability-indicating methods. Specifically, the purity of separated peaks was assessed using an orthogonal column in the second dimension, which was selected based on the hydrophobic subtraction model.[44]

Due to the obvious benefits that 2D-LC offers in several applications of pharmaceutical analysis, it is reasonable to think that its implementation in pharmaceutical laboratories will be wider and of the utmost interest in the near future. However, it is also clear that LC-MS (involving benchtop MS devices as the ones reported in Section 7) should not be neglected in such applications, as it can be considered a 2D analytical procedure. Finally, it is worth mentioning that several 2D-LC approaches have also been applied in biopharmaceutical analysis due to the structural complexity of the samples. However, for the sake of clarity, relevant examples related to this field will be discussed in Section 6.

4. Supercritical fluid chromatography of drugs

Supercritical fluid chromatography (SFC) is an analytical technology originally developed in the 1960s. When pressurizing and heating some fluids beyond their critical point (supercritical state), they exhibit unique behavior as chromatographic eluents.[45] Indeed, the viscosity and diffusivity of such fluids are very close to those of a gas, resulting in high kinetic performance at modest pressures, while the density and solvating power are equivalent to that of a liquid, providing good solubility of the analytes. Despite these benefits, the

pharmaceutical industry showed limited interest in SFC early on and continued to use well-established GC and LC techniques. Thus, SFC is often perceived as a niche technique and remains a reference technique for chiral separations and preparative scale applications[46] in the pharmaceutical industry. However, in the last five years, [47] the technique has seen real change and a renewed interest for use in analytical achiral applications in the pharmaceutical analysis community. This was mainly driven by *(i)* significant modifications of the mobile-phase components and compositions, *(ii)* introduction of innovative stationary-phase chemistries and dimensions, and *(iii)* commercialization of state-of-the-art systems with a design that is based on recent UHPLC instruments, thus offering drastic improvements in instrument reliability and performance.[48] In this section, the applicability of SFC in pharmaceutical analysis, including the determination of drugs in formulations and biofluids, is critically discussed.

One of the most significant changes in SFC, from its early inception to modern times, has taken place in the mobile-phase components and compositions.[48] In particular, the inclusion of organic solvents and the use of additives (acids, bases or salts) has dramatically extended the range of applicability of SFC to include more polar and ionizable compounds. In this context, Galea *et al.* investigated the possibility of blending several organic solvents (i.e., ethanol, methanol, isopropanol, THF, acetonitrile) rather than using only methanol (most common solvent) to further improve the selectivity of SFC during the method development of drugs. However, the nature of the modifier leads to only minor changes in terms of the elution order, retention and selectivity in comparison with changes to the stationary phase[49] of achiral SFC. West *et al.* have recently investigated the effects of additives on SFC drug analysis. They demonstrated that additives in the usual concentration range (0.1% or 10 – 20 mM) do not modify the polarity in the immediate vicinity of the probe.[50] They also used color indicators to determine an apparent pH of 5 for carbon dioxide–methanol mobile phases. They finally showed that adding a basic additive had little influence on the apparent pH, while acidic additives can further decrease the pH below 1.7.

In recent years, several groups have published works related to finding the ideal sample diluents for modern SFC. If the sample diluent is adequately selected, it becomes possible to inject larger volumes in SFC, leading to a substantial improvement in sensitivity,[51] which is particularly relevant for the impurity profiling of drugs. As illustrated in Figure 6, it was demonstrated that aprotic solvents, such as methyl tert-butyl ether, dichloromethane, acetonitrile or cyclopentyl methyl ether, are well suited for the injection of large volumes in modern SFC, while MeOH was generally the worst option. However, the stationary-phase chemistry may also have a strong impact, and some of these diluents did not perform equally well on each column. This was probably related to competitive adsorption of the analyte and the diluent onto the stationary phase, as described elsewhere.[52,53] Therefore, the sample

diluent must be selected not only based on the nature of the analytes but also on the column chemistry.

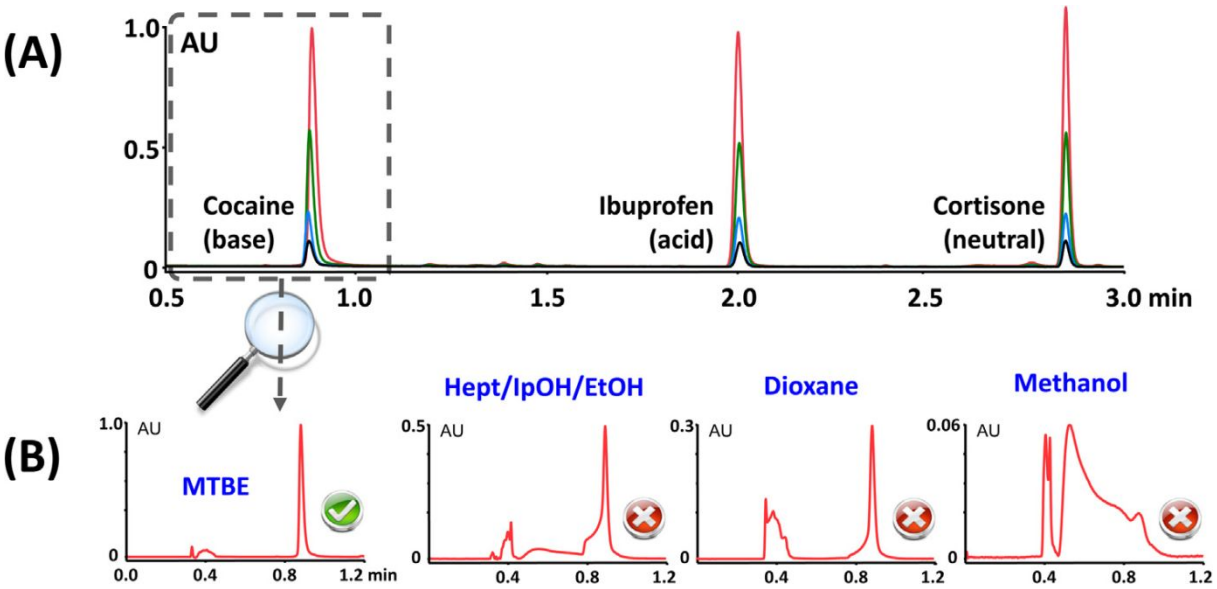

Figure 6. (A) Chromatograms obtained from the injection of 1 (black), 2 (blue), 5 (green) and 10 (red) µL of a mixture of cocaine, ibuprofen and cortisone at 100 µg/mL each in MTBE on 2-PIC. (B) Injection of 10 µL of a cocaine solution at 100 µg/mL with four different diluents (MTBE, heptane/IpOH/EtOH, dioxane and MeOH). Reprinted from J. Chromatogr. A, Vol. 1511, V. Desfontaine, A. Tarafder, J. Hill, J. Fairchild, A. Grand-Guillaume Perrenoud, J.L. Veuthey, D. Guillarme, A systematic investigation of sample diluents in modern supercritical fluid chromatography, pp 122-131.[51] Copyright 2017, with permission from Elsevier.

Since there is an increasing number of SFC practitioners who are transitioning from "old-fashioned" to modern SFC (also known as ultrahigh performance SFC, UHPSFC), which involves the use of shorter, thinner columns packed with smaller particles, there is a need to develop tools to "easily" transfer SFC methods. In LC, method transfer is quite straightforward and can be performed using geometric rules. However, in SFC, it is important to maintain the same average pressure (and therefore density) in the original and target systems to avoid, or at least limit, changes in retention and selectivity. Tarafder *et al.* recently worked on this issue[54,55] and suggested a solution to match average pressures for scaling over most of the operating conditions used in SFC. However, this work was only performed in an isocratic mode, and there would be a need for scaling rules in gradient modes, which are far more complex.

Another obvious benefit of SFC is the low mobile-phase viscosity, which facilitates the use of long columns. It is even possible to couple several complementary columns in series that offer different selectivities under SFC conditions. West *et al.* suggested combining Acquity, UPC^2 HSS C18 SB and Nucleoshell HILIC (zwitterionic phase) in series, as these two columns offer a high degree of complementarity.[56] To evaluate the potential of this approach, 25 individual drug substances containing various impurities were analyzed. It was shown that the configuration of two columns coupled in a single analysis was more informative than that

of two consecutive methods with the individual columns in approximately 35% of the cases, while the analysis times were nearly the same.

Because modern SFC systems are more reliable, robust and sensitive than previous generations of instruments, the analytical methods developed for SFC can be fully validated and used for the determination of impurities at low concentrations in pharmaceutical formulations. For example, an SFC method was recently developed for the determination of salbutamol sulfate and related impurities as an alternative to a European Pharmacopeia (*Ph. Eur.*) LC method involving ion-pairing reagents.[57] The separation was seven times faster (7 *vs.* 50 min) and was validated according to ICH Q2 guidelines using an accuracy profile approach. Then, the authors performed an inter-laboratory study, involving 19 participating laboratories across four continents and nine different countries, on the same sample to assess the reproducibility of the method and evaluate whether or not this chromatographic technique could become a reference method for quality control (QC) laboratories. Repeatability and reproducibility variances in the SFC were found to be similar to or better than those described for the LC methods, thus highlighting the adequacy of the SFC method for QC laboratories.[58]

Another important trend in SFC is its coupling to mass spectrometry (MS), since this detector has become the gold standard for the determination of trace compounds in complex matrices due to its high specificity, sensitivity and quasi-universality.[59] As shown in Figure 7, various robust and flexible SFC-MS interfaces have been proposed to *(i)* manage the compressibility of the SFC mobile phase when it is no longer under the control of the backpressure, *(ii)* improve the ionization yield in the ESI mode when the mobile phase has a large percentage of CO_2, and *(iii)* maintain the chromatographic integrity in terms of retention, selectivity and efficiency.[60]

Today, it is clear that the presence of splitter and make-up pumps in the interface are prerequisites to achieve a sufficient degree of flexibility and high detection sensitivity in ESI.[61] In this context, the pre-back pressure regulator (pre-BPR) splitter with a sheath pump interface (interface D in Figure 7), commercially available from Waters and Agilent, appears to be the most prevalent interface for SFC-MS. With this interface, the performance of SFC-MS has been assessed for a wide range of doping agents, including beta-blockers, diuretics, and stimulants. In addition to the excellent retention of the most polar drugs and its complementarity with RPLC, it appears that the sensitivity of SFC-MS/MS was equivalent to that of RPLC-MS/MS for 46% of the compounds and the sensitivity was improved in SFC-MS/MS for 32% of the compounds.[62]

In another study related to doping control analysis, most of the investigated anabolic agents, synthetic cannabinoids and glucocorticoids, were detectable at very low concentrations (below 0.1 ng/mL) in urine by SFC-MS.[63] In recent times, various research groups have

worked to evaluate matrix effects (MEs) in SFC-MS, since it remains one of the major drawbacks of quantitative MS-based bioanalytical methods. Desfontaine *et al.*[64–66] systematically compared the incidences of ME in RPLC-MS and SFC-MS for a wide range of drugs in urine and plasma matrices using three different SFC column chemistries with nonselective and selective sample preparation procedures. In the case of urine, a lower susceptibility to ME was systematically observed for SFC *vs.* RPLC. For plasma samples, the results strongly depended on the stationary-phase chemistry, and the MEs were often lower in RPLC than in SFC. Haglind *et al.* attributed MEs in SFC-MS to clusters of methanol present in the SFC mobile phase and alkali metal ions that are naturally abundant in biological matrices. These clusters were retained in SFC, causing significant ion suppression.[67] In another study, Fujito *et al.*[68] evaluated the MEs of different matrices (four different food matrices), compounds (pesticides) and SFC-MS interface designs. They observed no significant difference in the occurrence of matrix effects between SFC-MS and LC-MS.

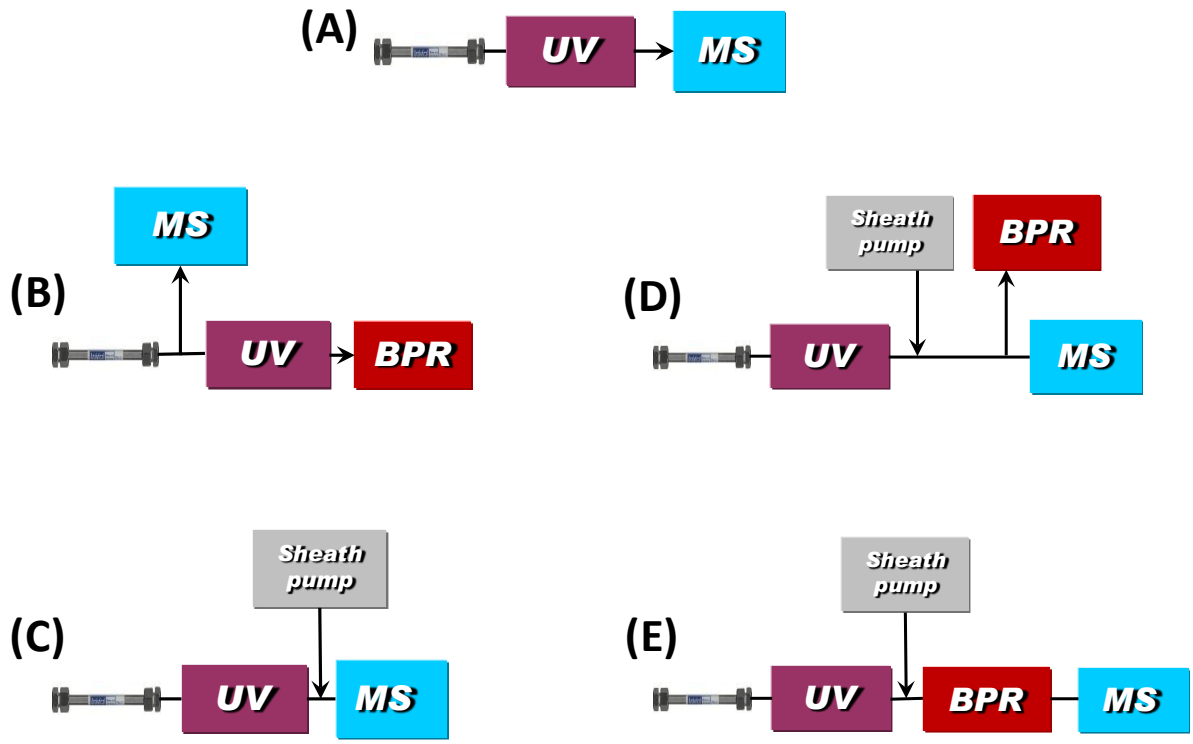

Figure 7. Schematic representations of the five most common SFC–MS interfaces. (A) "direct coupling" interface, (B) "pre-UV and BPR splitter without sheath pump" interface, (C) "pressure control fluid" interface, (D) "pre-BPR splitter with sheath pump" interface, and (E) "BPR and sheath pump with no splitter" interface. Reprinted from J. Chromatogr. B, Vol. 1083, D. Guillarme, V. Desfontaine, S. Heinisch, J.L. Veuthey, What are the current solutions for interfacing supercritical fluid chromatography and mass spectrometry?, pp 160-170 (ref [61]). Copyright 2018, with permission from Elsevier.

SFC has been used for decades for the analysis of highly hydrophobic substances (i.e., lipids, petrochemical samples) as an alternative to normal-phase LC due to the nonpolar characteristics of pure CO_2. In the last 10 – 15 years, the application of SFC has been

extended to include the analysis of moderately polar substances, such as drugs, by using up to 30 – 40% MeOH in the mobile phase. More recently, SFC has also been applied to the analysis of highly polar substances, thanks to the use of very high organic solvent proportions (up to 100%) and significant amounts of volatile salts in the mobile phase. In these cases, supercritical conditions are no longer observed, but this suggests that SFC has the ability to analyze mixtures with an extended polarity range, which includes nonpolar, polar and very polar compounds, in a single run when applying a gradient from pure CO_2 to pure methanol. In these particular conditions, the term SFC is not technically correct, since the mobile phase is no longer under a supercritical state. This approach has been addressed as unified chromatography (UC) or enhanced-fluidity liquid chromatography (EFLC) and is increasingly becoming widely used. Various applications have been reported for the simultaneous analysis of liposoluble and hydrosoluble vitamins,[69] the determination of numerous environmental pollutants having an extended range of polarity,[70] the simultaneous determination of lipophilic and hydrophilic metabolites (metabolomics),[71] the analysis of numerous pesticides[72] covering a wide polarity range, and, more surprisingly, the analysis of proteins (the achieved performance were, however, far from those reported in Section 6 using LC conditions).[73]

When taking into account the above mentioned benefits of SFC, we better understand the versatility of the technique and the fact that it can be used in a wide range of applications, as illustrated in some of the most recent work. Indeed, SFC has been applied to the analysis of a wide range of pharmaceutically relevant compounds, such as lipids, [74] steroids, [63,75–77] natural products, [78,79] liposoluble vitamins, [80–82] drugs, [83] inorganic anions and cations, [84] and drugs in biological matrices (bioanalysis).[85,86]

5. HILIC for polar and ionizable compounds

Hydrophilic interaction chromatography (HILIC) has emerged as a complementary technique to RPLC that is particularly suited for the analysis of polar and ionizable drugs, metabolites and other biologically relevant compounds (that are not sufficiently retained in RPLC). In HILIC, the stationary phase is hydrophilic, while the mobile phase is composed of a large percentage of polar aprotic solvent (acetonitrile) together with water and salts. The advantages of HILIC (i.e., orthogonality with RPLC, better retention of polar substances, low column backpressure, increased MS sensitivity) have been discussed several times in the past.[87] A large variety of biologically active substances, including amino acids, peptides, carbohydrates, neurotransmitters, oligosaccharides, nucleotides, and nucleosides, can be successfully analyzed by HILIC. However, adoption of this technique has been slowed by the experiences of users with poor reproducibility/robustness and unsuitable performance. Therefore, some research groups have recently increased their efforts to circumvent the

known limitations of HILIC, including the long equilibration times, poor peak shapes, and a limited understanding of the HILIC mechanism.

Notably, the re-equilibration times of the HILIC mode have been reported to be exceptionally long compared to those of the RPLC mode.[88] This has been attributed to the long stabilization time of the water layer at the surface of the stationary phase. McCalley recently performed a systematic study involving acidic, basic and neutral solutes, various stationary phase chemistries (i.e., bare silica, amide and zwitterionic) and different column storage conditions.[89] It was found that full equilibration could take up to one hour, but was strongly dependent on the nature of the stationary phase, storage-solvent conditions and mobile-phase flow rate. Of note, zwitterionic columns take longer to equilibrate than silica columns, which are known to have thinner water layers. As illustrated in this work, the selectivity of the separation noticeably varies based on the equilibration time, showing that it is necessary to fix the equilibration time to a set value in order to obtain consistent results. Although the time required for full equilibration might be considered a serious drawback in the gradient elution mode, repeatable partial equilibration can be achieved much faster (after a re-equilibration time of only 5 min, but it requires two preliminary conditioning runs on the column that had taken the longest amount of time to achieve full equilibration) while maintaining excellent retention time reproducibility.

The mobile-phase additive plays a key role in HILIC, since it allows for suitable peak shapes and low retention-time variability by stabilizing the charge of the ionizable solutes and ionogenic groups on the surface of the stationary phase.[90] Even when conventional acids (i.e., formic acid and acetic acid) have been used in HILIC, they do not provide sufficient ionic strength in high concentrations of ACN to produce good peak shapes.[91] Therefore, the majority of modern HILIC separations are conducted with ammonium formate and ammonium acetate buffers, with a mobile phase pH ranging between 3 and 6. Interestingly, Heaton *et al.* demonstrated that the use of a citrate buffer, rather than an ammonium formate buffer, at pH 3 improved the peak shapes of catecholamines due to a reduction in the negative effects of metals in the system by their preferential complexation.[92] More recently, retention studies were also performed at a pH below 3 using either TFA or stronger ion-pairing reagents, such as heptafluorobutyric acid and methanesulfonic acid. Some unexpected behavior was noticed, and in particular, TFA use resulted in anion exchange properties, which contrasts with the cation exchange typically found when using an ammonium salt buffer.[93] Therefore, when using 0.1% TFA as the mobile phase additive, the elution order of drugs was very different from that observed with ammonium formate at pH 3.

The nature of the sample diluent is another important parameter that needs to be adequately controlled to achieve sharp peaks in HILIC. This was demonstrated some years ago by Ruta

et al.[94] and continues to attract attention today.[92] To limit deterioration of the column efficiency, the sample should ideally be diluted in a high proportion of aprotic solvent (at least 80% ACN). Small increases in the water content of the injection solvent may be acceptable, if the injection volume remains low (below 1% of the column volume), to accommodate compounds that are poorly soluble in an acetonitrile-rich mobile phase. Obviously, the detrimental effects of increased injection volume and solvent mismatch were more serious for solutes with smaller retention factors.[92] Recently, Gritti *et al.* highlighted the importance of the sample diluent in the analysis of cetirizine (a second-generation antihistamine drug) when using a recommended USP HILIC method.[95] Indeed, a significant peak deformation was noticed due to the higher concentration of a strong solvent (water) in the sample diluent compared to the mobile phase. The concentration profiles of cetirizine and water were calculated using the equilibrium dispersive model of chromatography. Based on this work, it appears that various modifications should be brought to HILIC methods to maintain peak-shape integrity. First, the water concentration of the diluent should not exceed that of the mobile phase. If that is not possible, the retention factor of the analyte should be at least 2-fold smaller than that of the water perturbation. If this is not possible, the amount of pre-column sample dispersion should be increased until the decrease in efficiency is too great. Finally, if none of the above mentioned technical solutions works, the injection volume can be decreased, as long as the impurities remain detectable.

To further extend the applicability of HILIC to a wider range of pharmaceutical compounds, various new HILIC columns have appeared in research papers every year.[87,96] Silica-based materials remain, by far, the most widely used stationary phases for HILIC. In particular, bare and amide-bonded silica, zwitterionic and diol groups are the most common phases used to perform HILIC separations of pharmaceutically relevant compounds. HILIC phases can be intuitively categorized based on their possible ionic interactions, including neutral (e.g., amide, cyano, diol), positively charged (e.g., amino, imidazole, triazole), negatively charged (e.g., polyaspartic acid, bare silica) and zwitterionic (e.g., sulfobetaine or peptide) phases.[87] In addition to the ionic selectivity, the hydrophilic selectivity also plays an important role and depends on the thickness of the water layer. For instance, the amide and zwitterionic phases possess large water layer thicknesses, while bare silica possesses the smallest.[59] In addition to these well-known stationary phases, some additional HILIC stationary phases were recently described. A novel polyacrylamide-based silica stationary phase was prepared by Cai *et al.,*[97] using a two-step synthesis method, for the analysis of carbohydrates using alcohols as the mobile phase rather than acetonitrile (thanks to the high hydrophilicity of this phase), which was beneficial for green analysis and the purification of polar compounds. A multifunctional phase, synthesized from L-isoleucine and 4-phenylbutylamine, was used as a mixed-mode HILIC/RPLC phase.[98] This new stationary phase was found to be particularly

well suited to the analysis of nucleotides/nucleosides and for the simultaneous analysis of weakly polar and nonpolar solutes. Hou *et al.*[99] prepared a porous graphitic carbon (PGC) stationary phase with quaternary ammonium-polyvinyl alcohol-mixed functional groups for HILIC operation. This phase offered typical HILIC characteristics but exhibits very different selectivity compared to other HILIC phases and bare PGC. In addition, this phase was superior to silica-based HILIC phases in terms of pH tolerance (2.1 – 12.7) and bleeding. Qian *et al.*[100] also suggested a valuable solution to reduce the column bleeding that results from silica dissolution. For this purpose, multiple layers of a polyvinyl alcohol cross-linked with glutaraldehyde were coated onto silica gel, leading to a 20-fold reduction in bleeding compared to bare silica.

When combining HILIC and MS, a substantial average increase in the sensitivity by a factor of 7 to 10 can be expected compared to RPLC-MS.[101] This sensitivity increase of HILIC was obviously explained by the difference in acetonitrile concentration during the elution of drugs (on average, 30% ACN in RPLC and 80% ACN in HILIC), leading to better analyte desolvation. However, this high proportion of organic solvent also favorably influenced ionization by modifying the mobile-phase pH and solute pK_a. As such, the ionic character of the analytes in solution also plays an important role in explaining the sensitivity enhancement of HILIC. However, the sensitivity improvement of HILIC is strongly dependent on the design of the ESI source.[87] In a recent systematic study, a large majority of pharmaceutical compounds were detected better with HILIC *vs.* RPLC when using a Waters MS. On a modern Agilent triple quadrupole instrument, the performance drastically depended on the mobile-phase pH and flow rate conditions, and the benefits of HILIC were only noticed at low flow rates and intermediate pH (pH = 6). Surprisingly, on the Sciex instrument, most of the drugs were detected better with RPLC *vs.* HILIC. As a general comment, the increase in sensitivity observed in HILIC was less significant with recent LC–MS platforms than with old-generation instruments. Indeed, the improved ESI sources used in newer mass analyzers allow for enhanced evaporation efficiency, and this feature is particularly beneficial for RPLC conditions. In addition to sensitivity, matrix effects in RPLC and HILIC were also systematically studied using a wide range of model pharmaceutical compounds in plasma and urine matrices. When using a nonselective extraction procedure (dilute and shoot or protein precipitation), matrix effects were lower in RPLC *vs.* HILIC regardless of the matrix. In contrast, HILIC appears to be a valuable alternative to RPLC for plasma and urine samples treated using a selective extraction procedure.[102] More importantly, the compounds influenced by the matrix effects were different in HILIC and RPLC, suggesting that changing the chromatographic method can be an interesting strategy to reduce/eliminate matrix effects in bioanalysis.

In terms of pharmaceutical applications, HILIC is currently used for the analysis of polar substances that cannot be successfully analyzed by RPLC, such as anticancer drugs (cisplatin[103] and 5-FU[104]). It is also a reference method for the analysis of carbohydrates, [105,106] amino acids,[107] small peptides[108] and glycopeptides.[109] It can also be a suitable replacement strategy for ion exchange in the analysis of small inorganic ions.[110] However, progress in HILIC is clearly driven by the needs of bioanalysis and, above all, metabolomics,[111] where HILIC has been established as a reference strategy. In bioanalysis, HILIC can help in analyzing polar substances in biological matrices.[112,113] It allows for sufficient retention of such substances and high sensitivity with ESI/MS. In metabolomics, where the purpose is to comprehensively study the low molecular weight compounds present in cells, organs, and organisms, both RPLC and HILIC are employed to cover a broad range of the metabolome. In particular, HILIC is an essential technique because of the significant presence of polar compounds in the metabolome and the need to improve the detection of these polar metabolites. In recent years, despite the known limitations of HILIC (i.e., poor retention time reproducibility, analytical drift with analysis of multiple samples), HILIC-MS has been successfully used to analyze a wide range of metabolites[114] (also including some lipids[115]) in complex matrices such as plasma, urine,[116] cell extracts,[117] or cerebrospinal fluids.[118] As an example, Figure 8 shows the coverage of various metabolites, including phosphates, sugars, amino acids and organic acids, by various RPLC and HILIC methods.

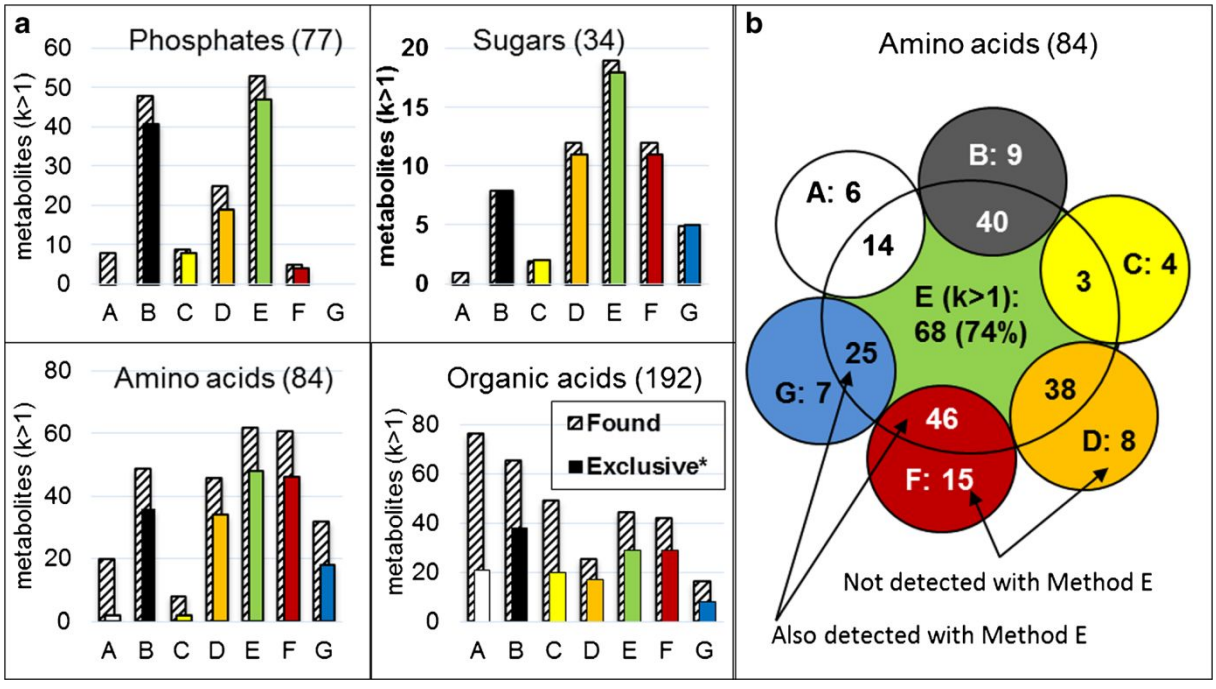

Figure 8. Method A: C18 RPLC column, method C: mixed mode HILIC/RPLC column, methods B and E: zwitterionic HILIC column, Method D and F: neutral HILIC column, method G: amino HILIC column. (a) Coverage of metabolite groups. Shaded bars: retention factor >1, good peak shape. Colored bars: number of metabolites not captured by RP C18 (method A). For method A, "exclusive" compounds are not detected by methods B–F. (b) Complementarity of phosphorylcholine-based column E and other HILIC columns for the amino acid subgroup. Adapted from Anal. Bioanal. Chem., Vol. 408, S. Wernisch, S.

Pennathur, Evaluation of coverage, retention patterns, and selectivity of seven liquid chromatographic methods for metabolomics, pp 6079-6091.[114] Copyright 2016, with permission from Springer.

6. Analysis of protein biopharmaceuticals and conjugates

Biopharmaceuticals are therapeutic proteins produced *in vivo* through recombinant DNA technology and are generally used for the treatment of severe diseases, such as cancer, autoimmune disorders and cardiovascular diseases.[119] Although several kinds of therapeutics fall within the category of protein biopharmaceuticals (hormones, growth factors, blood factors, vaccines, anticoagulants, cytokines and others), monoclonal antibodies represent the largest percentage of these drugs (mAbs), followed closely by mAb-related products, such as antibody-drug conjugates (ADCs), fusion proteins and then bispecific antibodies (bsAbs). The success of these drugs compared to other biopharmaceuticals is due to their ability to be used in immune checkpoint therapy,[120] by targeting the regulatory pathways of T cells to enhance the antitumor immune response and, therefore, provide a new weapon against cancer. Immune checkpoint therapy has led to the most important clinical advances of modern times, and its pioneers James P. Allison and Tasaku Honjo have been awarded the 2018 Nobel Prize in Medicine.[121]

Beyond the revolutionary mechanism of action, mAbs and its related products manifest an undeniable analytical complexity related to their size and structural microheterogeneity, mainly through posttranslational modifications (PTMs). Consisting of large immunoglobulin G (IgG) proteins of approximately 150 kDa, mAbs exist as proteoform ensembles instead of one unique molecule. Although this unique behavior is due to their being synthesized by living organisms and cannot be avoided, it needs to be strictly controlled and characterized. In this context, chromatography plays a primary and pivotal role, since a plethora of different analytical methods (electrophoresis, spectroscopic, mass spectrometry and chromatography) are required to discover the identity, purity, stability and safety of mAbs and related products. In fact, a single technique is incapable of fulfilling the different analytical demands, and several efforts have been made in recent years to ensure analytical tools evolve in-line with the structural complexity, such as purposeful design of stationary phases and bioinert systems and materials (*vide infra*). Interested readers are encouraged to consult a series of LC mode-dedicated reviews provided by Fekete and coworkers that discuss the analytical solutions of state-of-the-art RPLC, [9] size exclusion chromatography (SEC),[122] ion-exchange chromatography (IEX),[123] and hydrophobic interaction chromatography (HIC)[124] for mAbs analysis, while general analytical approaches have been reviewed by Beck *et al.*[125–127]

As shown in Figure 9, different levels of LC analysis are possible, namely, the intact, subunit (also referred to as the middle-up approach), peptide, amino acid, and glycan levels. Sample

pretreatment is generally not required for the analysis of mAbs and related products at an intact level, since native features of the biomolecules are investigated at this stage of the analysis.[128,129] In this context, non-denaturing LC modes such as SEC, cation-exchange (CEX), and HIC are applied to determine information on the size, charge, and hydrophobic variants, respectively.[128] Denaturing techniques such as RPLC and HILIC are instead applied to the subunit, peptide, amino acid, and glycan levels to complete the detailed analytical characterization and determine the information of the PTMs.[130] In this context, coupling of LC to MS has obtained crucial significance in determining protein identity and the punctual characterization of PTMs, generally performed through peptide mapping analysis. Sample pretreatment is generally used at these stages of the analysis to obtain streamlined access to the subunits, generate peptides and amino acids or specifically release glycans for profiling.[131]

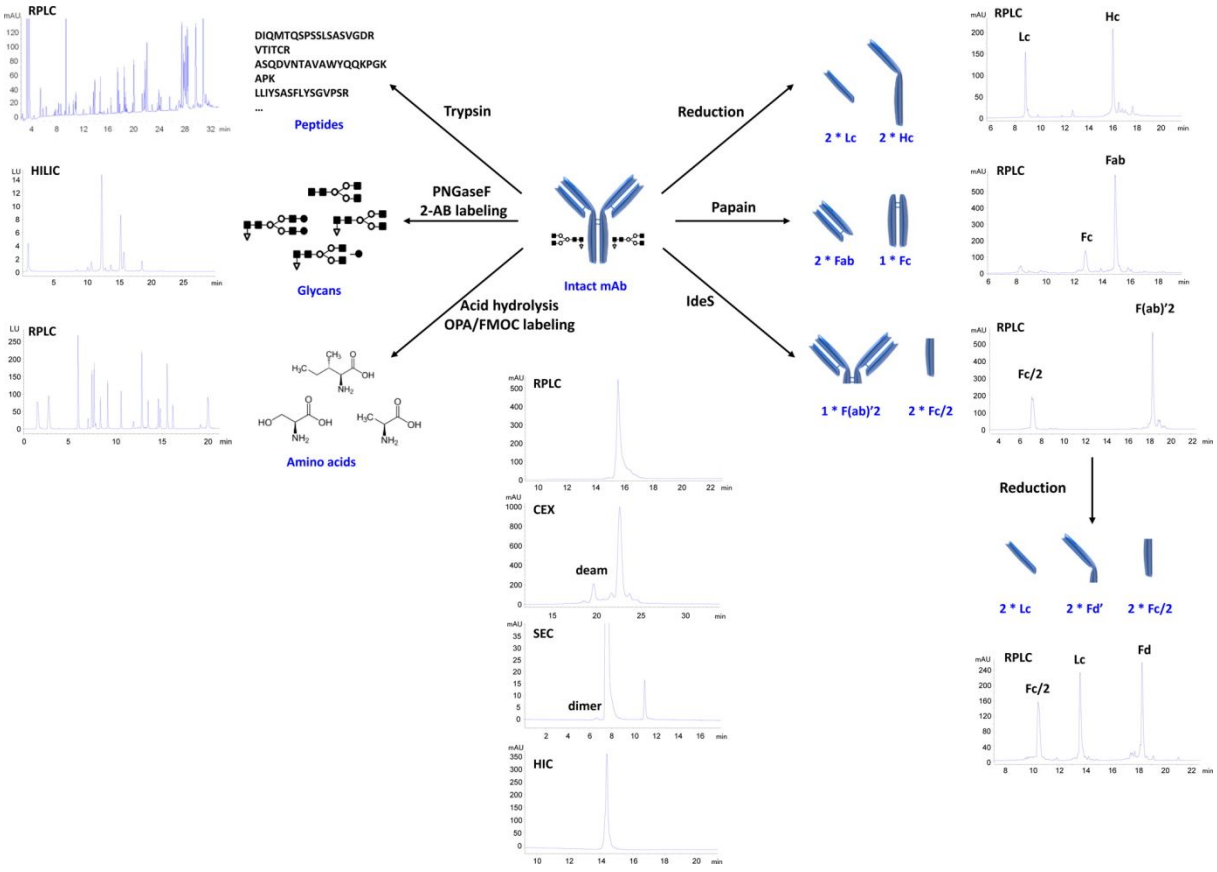

Figure 9. Flowchart of the different LC approaches (RPLC, HIC, SEC, CEX, and HILIC) that are generally used for mAb characterization (trastuzumab as an example) at the protein, peptide, glycan, and amino acid levels. Reproduced from Fekete, S.; Guillarme, D.; Sandra, P.; Sandra, K. Chromatographic, Electrophoretic, and Mass Spectrometric Methods for the Analytical Characterization of Protein Biopharmaceuticals, Anal. Chem. 2016, 88 (1), 480–507 (ref [119]). Copyright 2016 American Chemical Society.

As anticipated, particular attention has recently been devoted to the “bioinertness” of LC systems and materials used for biopharmaceutical analysis. Indeed, a general issue in protein analysis is the adsorption of the sample onto the HPLC instrument, at the injector,

tubes or detector cell. A possible solution to this issue is the replacement of the hydrophobic polyether ether ketones (PEEK) used for the chromatography tubing and injection needles with more inert materials such as titanium, stainless steel or PEEK-Sil (fused silica inside, PEEK outside), even though fused silica and stainless steel might not completely eliminate protein adsorption. Another more recent option is to use purposely designed bioinert LC systems and materials.[128] In this context, several manufacturers have included in their commercial offerings Bio-UHPLC instruments specifically designed to answer the needs (and solve the issues) of biomolecule analysis.[132] Some examples include the 1260 Infinity Bio-inert HPLC system from Agilent Technologies (equipped with an iron- and steel-free solvent delivery module, assuring a metal-free sample-contacting surface that minimizes undesired surface interactions), and the ACQUITY UPLC H-Class PLUS Bio system from Waters, also engineered with bioinert iron-free flow paths and materials, assuring better sample recovery and minimal carryover, regardless of whether the chromatographic mode is RPLC, IEX, SEC or HIC. Other recent bioinert systems to mention are the metal-free Prominence Inert LC system from Shimadzu, ideal for analyzing aggregates in biopharmaceuticals, and the UltiMate 3000 BioRS system from ThermoFisher Scientific, equipped with stainless steel-free flow paths and claiming a near-zero dead-space volume.

The main goal of this review was to provide an overview of the recent advances in LC biopharmaceutical analysis, and therefore, a selection of the latest progress will be described hereafter, including the application of 2D-LC, new SEC and RPLC column technologies, and HILIC mode performed at the protein level.

As anticipated in Section 3, 2D-LC has been widely applied to the characterization of protein biopharmaceuticals, especially for mAb and ADC structural characterization at the peptide, subunit and intact levels. The application of comprehensive 2D-LC for peptide mapping of mAbs in both R&D and routine (QA/QC) environments was discussed by Vanhoenacker and coworkers.[133] A tryptic digest of trastuzumab was analyzed by 3 different 2D-LC combinations, including CEX × RPLC, RPLC × RPLC, and HILIC × RPLC, with both UV (DAD) and MS detection. The orthogonal information obtained by the application of the different LC × LC approaches allowed for assessing both the identity and purity of the sample. In addition, comparison studies were performed between two different batches of trastuzumab and a biosimilar agent under development that highlighted some differences, such as the increase of a specific deamidation from 8 to 13% and the clipping of the C-terminal lysine that was almost driven to completion in the originator but only removed in 87% of the biosimilar molecules. Based on the precision of the method in terms of the peak volume and retention time, the authors were confident about using the approach in future QA/QC testing. Comparisons of originator and biosimilar mAbs were also performed by Sorensen and coworkers at the middle-up level of analysis.[134] Specifically, 3

reference/biosimilar pairs of mAbs (i.e., trastuzumab, cetuximab and infliximab) were analyzed by CEX × RPLC–HRMS, after which mAb subunits were obtained by either *IdeS* digestion or *IdeS* digestion followed by reduction with dithiothreitopl (DTT). Interestingly, despite the size of the subunits, 23 chemically unique mAb fragments were detected in a single sample. Furthermore, direct comparison between the 2D counterplots allowed for a facile assessment of the degree of similarity between the reference and biosimilar samples.[134] As an extension of this work, in a very recent report, the authors applied HILIC × RPLC–HRMS to the separation of mAbs at the subunit level to reveal the extent of glycosylation on the Fc/2 and Fd subunits with analysis times on the order of 2 h. In comparison to previous CEX × RP separations of the same molecules, chromatograms from the HILIC × RPLC-HRMS separations were found to have higher resolution and revealed separation of some of the glycoforms that coeluted in the CEX × RP separations.[135] A more general overview of the potential benefits of 2D-LC over conventional 1D-LC for characterizing therapeutic mAbs is provided by Stoll *et al.*,[136] where the possibility to increase the resolving power, obtain complementary information from different column selectivities and directly couple the 2D-LC system to MS are extensively discussed. In addition, the suitability of 2D-LC to the characterization of more complex samples, such as ADCs, is anticipated. In this context, several contributions have arisen in recent years, in response to this analytical demand, using heart-cutting, [137] multiple heart-cutting, [138] and comprehensive 2D-LC.[139] HIC × RPLC–HRMS was performed by Sarrut *et al.*[139] to obtain and profile the drug-to-antibody ratio (DAR) of brentuximab vedotin in the first dimension (HIC) with an in-line desalting step performed in the second dimension (RPLC) prior to the coupling with MS that allowed accurate identification of positional isomers. Based on the same approach, Ehkirch and coworkers[140] developed a 4-dimensional platform, consisting of HIC × SEC–IM–MS, for performing in-line characterization of brentuximab vedotin in native conditions. In this configuration, the second dimension (SEC) was exclusively used as a fast desalting step to maintain the non-denaturing conditions of the separation performed in the first dimension (HIC) prior to the accurate identification performed by the IM–MS.

Recently, the thorough and simultaneous characterization of both small and large molecules (drug payloads decorating mAbs in ADC formats) has also stimulated interest in the applications of 2D-LC.[136,137,139] As practical examples, Venkatramani *et al.*[141] performed qualitative and quantitative analysis by high-resolution sampling (HRS) 2D-LC of a key linker drug intermediate used in ADCs. The approach was applied to three groups for comparison and was able to identify 15 impurities coeluting with the linker drug intermediate in the first dimension that were then separated in the second dimension. Similarly, Li and coworkers[142] applied heart-cutting SEC–RPLC to identify and quantify unconjugated small-molecule drugs

and their related small-molecule impurities in ADC samples without the need to perform additional sample preparation. Sandra *et al*.[143] demonstrated the versatility of heart-cutting and comprehensive 2D-LC for mAb clone selection during mAb and biosimilar molecule development. In this work, the authors combined Protein-A affinity chromatography (first dimension) with SEC, CEX, or RPLC (second dimension), with the goal of simultaneously assessing mAb concentration and critical structural aspects such as aggregation, fragmentation, charge heterogeneity, molecular weight (MW), amino acid sequence and glycosylation. Data were first acquired at the intact protein level in LC–LC mode and then complemented by information derived from peptide mapping performed in LC × LC mode coupled to MS. The versatility of the approach was demonstrated through the analysis of trastuzumab and tocilizumab-producing CHO clones and has the potential to be extended to process optimization.

Beyond applications of 2D-LC MS, another recent advance in LC analysis of biopharmaceuticals has occurred thanks to the introduction to the market of new SEC, RPLC, and HILIC column technologies.

As one example, in recent years, we have been witnessed the translation of SEC methods into ultrahigh performance SEC (UHP-SEC). This has been possible through a focus on column modifications in terms of the particle size, inertness, and column dimensions, resulting in small inert SEC columns packed with sub-3 μm particles. UHP-SEC columns meeting these specifications are currently available from various providers, namely, the AdvanceBio SEC column (2.7 μm, 300 Å) from Agilent, the TSkgel UP-SW3000 column (2.0 μm, 250 Å) from Tosoh, the Yarra SEC-X150 (1.8 μm, 150 Å) and the SEC-X300 columns (1.8 μm, 300 Å) from Phenomenex, the Unix (1.8 μm, 200 Å) and the Unix-C columns (1.8 μm, 300 Å) from Sepax, and the ACQUITY UPLC Protein BEH SEC column (1.7 μm, 200 Å) from Waters. The practical possibilities and limitations of a few of the above mentioned columns in the field of biopharmaceutical analysis were recently evaluated by Goyon and coworkers,[144] by analyzing several commercial mAbs and ADCs. It was found that the UHP-SEC columns were equivalent in terms of mAb characterization performance, since the average resolution for each column was between 1.9 – 2.1 for the monomer and dimer forms of 10 different commercial mAbs. Furthermore, a drastic increase in the throughput of aggregate analysis was achieved, with analysis times ranging between 3 and 8 min. Despite these kinetic advantages, it appears that uncontrolled secondary interactions still exist with these phases and affect both the elution times and the aggregate recovery. Among these secondary interactions, hydrophobic interactions were generally more prominent than electrostatic interactions, even though fine-tuning of the mobile-phase composition helped to mitigate these effects while maintaining non-denaturing conditions. However, it should be

noted that secondary interactions in UHP-SEC columns are much less pronounced compared to old-fashioned SEC columns packed with 5-10 µm particles. In this context, UHP-SEC columns demonstrated the ability to properly resolve the HMWS of highly hydrophobic structures, such as ADCs, without the need to add organic solvents to the mobile phase.[144] As a side note, having more inert SEC stationary phases may open the doors to a completely new set of applications, including the possibility of direct SEC–MS coupling. This possibility was indeed investigated by comparing UHP-SEC separations obtained with standard (nonvolatile) SEC mobile phases, consisting of 50 mM potassium phosphate and 250 mM potassium chloride, and MS-compatible (volatile) mobile phases, consisting of 100 mM ammonium acetate.[145] Thirty therapeutic proteins, including mAbs, ADCs, Fc-fusion proteins and a bispecific antibody (bsAb), were investigated. However, despite great expectations, it was shown that only the acidic therapeutic proteins (pI < 7) showed comparable separation behavior between the nonvolatile and volatile mobile phases, presumably as a result of the secondary interactions (hydrophobic and electrostatic) still playing an active role in the separations. Nonetheless, the ability of UHP-SEC columns to discreetly function with MS-compatible mobile phases was encouragingly applied as a "helping tool" when performing MS analysis in non-denaturing conditions.[146] Indeed, in the field of native MS, a major drawback is the manual sample preparation to desalt the product prior to MS analysis. Ehkirch and coworkers[146] provided a solution to this issue by suggesting the use of UHP-SEC as an in-line automated buffer exchanger. Interestingly, native MS performed with in-line desalting through UHP-SEC resulted in high-resolution mass spectra and improved mass accuracies compared to manual buffer exchange procedures.

There have also been some recent advances in RPLC column technologies for protein biopharmaceutical analysis. As discussed in Section 2, the advent of superficially porous particle (SPP) technology represented a real revolution in terms of column efficiency at low backpressures. Large biomolecules, such as therapeutic proteins, which are generally affected by slow diffusion rates, have benefited from the evolution of SPP technology towards the optimization of particle size, pore sizes, and shell thickness. Specifically, wide pore SPP materials with large particle and pore sizes but thin shells (3.5 µm, 450 Å, and 0.25 µm, respectively) showed optimal resolution for mAb separation[147] and were specifically commercialized for mAb analysis (AdvanceBio RP–mAb from Agilent). More recently, a wide pore silica-based SPP material with a high coverage of phenyl bonding has been proposed by Waters and commercialized as the BioResolve RP mAb Polyphenyl phase. This new material consists of medium particle sizes, large pore sizes and moderately thin shells (2.7 µm, 450 Å, and 0.40 µm, respectively) and was proposed as a novel surface chemistry to *(i)* limit silanol interactions, *(ii)* facilitate desorption, and *(iii)* improve resolving power. The performance of this novel RPLC phase was evaluated by Bobaly and coworkers[10] and

systematically compared to modern wide-pore phases possessing different structures, morphologies and chemistry. A special emphasis was placed on its unique, high coverage of phenyl bonding sites, which are able to offer an alternative hydrophobic interacting surface (steric effects and eventually π–π interactions), yielding advantageous selectivity for mAb subunit peaks and ADC species.[8,10] In a subsequent study, Bobaly *et al.*[148] discussed the ability of the BioResolve RP mAb Polyphenyl column to allow protein RPLC analysis under milder conditions with respect to current trends (80 – 90 °C and up to 0.1% TFA). Twenty-three mAbs were analyzed at temperatures ranging from 60 to 90 °C using various proportions of TFA and FA. The results demonstrated that temperature could be decreased to 75 °C for intact mAb and 65 °C for subunit mAb analysis, while maintaining a desirable peak shape and more than 90% recovery of the protein. Interestingly, in light of MS coupling, the phenyl bonded stationary phase in combination with mobile phases with either 0.03% TFA/0.07% FA or 0.1% TFA offered similar separation performance with, however, the non-negligible advantage of an increase in MS sensitivity of approximately 40% compared to a mobile phase of 0.1% FA.

Finally, yet importantly, recent advances in HILIC column technology also trigged unexpected LC applications in biopharmaceutical analysis. In fact, with the commercialization of wide pore HILIC amide phases (ACQUITY UPLC Glycoprotein BEH Amide from Waters and AdvanceBio Glycan Mapping from Agilent), the possibility of performing protein glycoprofiling at both intact and subunit (middle-up) levels became a reality. In fact, HILIC has generally been considered to be the gold standard for the profiling of released glycans in mAb analysis, but thanks to these new purposely-designed wide pore amide phases, its range of applications has been interestingly widened.

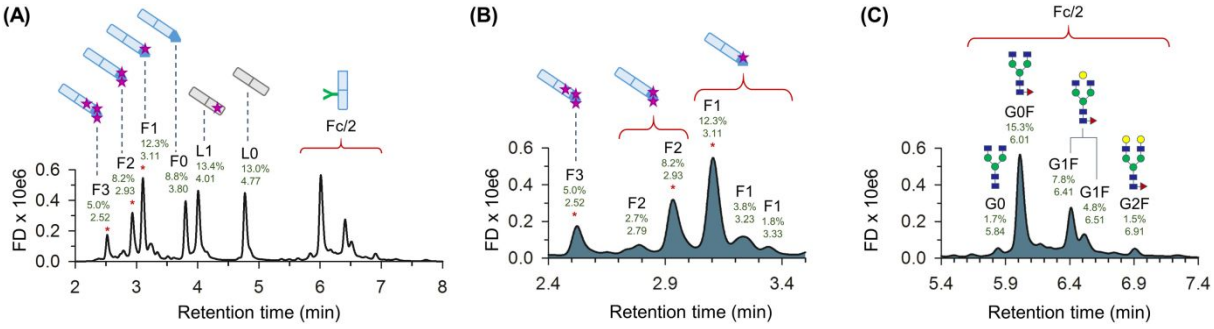

Figure 10. HILIC-MS middle-up analysis of brentuximab vedotin (A) and zoomed in view on the retention windows showing the elution of the drug-loaded Fd′ subunits (B) and the Fc/2 glycosylated fragments (C). Peak assignment is accompanied by the relative percentage peak area and retention time. Reprinted from J. Chromatogr. B, Vol. 1080, D'Atri, V.; Fekete, S.; Stoll, D.; Lauber, M.; Beck, A.; Guillarme, D. Characterization of an antibody-drug conjugate by hydrophilic interaction chromatography coupled to mass spectrometry, pp 37-41 (ref [149]). Copyright 2018, with permission from Elsevier.

The first application of HILIC performed at the intact and subunit levels was proposed by Periat and co-workers[150] through the analysis of relevant samples including insulin, interferon α-2b and a mAb, namely trastuzumab. Results showed high orthogonality between HILIC

and RPLC separation in terms of the elution order and selectivity but with the unique feature of HILIC to resolve glycoforms at the subunit level (fragments with sizes of 25–100 kDa). Consequently, D'Atri and co-workers investigated the feasibility of the HILIC middle-up approach in several other applications, such as *(i)* comparison of the glycosylation pattern between originator and biosimilar mAbs,[151] *(ii)* batch-to-batch comparison or clone monitoring,[152] and *(iii)* simultaneous glyco- and drug-profiling in ADC analysis.[149] In the former case, three pairs of originator/biosimilar mAbs (trastuzumab, infliximab and cetuximab) were analyzed with both wide pore RPLC and HILIC amide materials by middle-up analysis. The ability of HILIC to resolve hydrophilic variants facilitates both glycan profiling comparison and MS identification. Conversely, the same hydrophilic variants were not separated by RPLC and co-eluted.[151] Similarly, the benefits of the middle-up HILIC approach over RPLC were demonstrated through the analysis of brentuximab vedotin, an ADC consisting of a mAb armed with a cytotoxic drug payload, covalently bound to cysteine residues involved in inter-chain disulfide bonds. As shown in Figure 10, the drug payload and glycan modifications of this ADC were simultaneously characterized using a unique LC-MS middle-up analysis, demonstrating that HILIC is an effective and complementary analytical technique to RPLC for subunit-level characterization of immuno-conjugates.

7. Combination of modern LC with simple/low cost MS devices

Mass spectrometry is an essential technique in the pharmaceutical field, being of the utmost importance in several areas of product discovery, formulation, analysis, development and manufacturing. While high-resolution mass spectrometry (HRMS) is required for accurate structural elucidation, low-resolution mass spectrometry (LRMS) is useful in routine analysis applications as a support to pharmaceutical process chemistry, drug discovery and early development. Notably, LRMS analyzers are prone to miniaturization and thus more inclined to serve the need of having a "benchtop size" MS system. In this context, a comprehensive list of compact mass spectrometers was recently reported.[153,154] Among others, compact MS systems suitable for direct coupling to chromatographic systems for benchtop use included the single quadrupole mass analyzers 4500 MiD from Microsaic Systems,[155] the Expression series from Advion,[156] and the ACQUITY QDa Detector from Waters.[157] The above-mentioned MS detectors are all equipped with an ESI source, allowing positive and negative ionization modes in sequential analysis (4500 MiD and Expression) or the ability to cycle through four different acquisition modes within a single run (ACQUITY QDa).[154] Full scan MS or selected ion monitoring (SIM) can be selected as acquisition modes with an average mass range covering 40-1280 *m/z* and the ability to uphold LC flow rates up to 500 µL/min (Expression), 1000 µL/min (ACQUITY QDa), and 2000 µL/min (4500 MiD).

The possibility of fitting MS into the chromatography workflow at the benchtop level has

represented a real breakthrough in separation science, finding many applications especially in the field of pharmaceutical analysis. As an example, compact benchtop MS systems can be used for reaction monitoring as support to pharmaceutical process chemistry and drug discovery. In fact, the presence of reaction starting materials, products and intermediates can be simply followed at unit mass resolution in either the full scan or SIM mode. Some examples reported in the literature include the monitoring of salicylic acid formation, the quantitative analysis of phenylalanine, tyrosine, tryptophan and kynurenine in a rat model for tauopathies[158] and the monitoring of cross-coupling reactions.[159]

Another application of LC-MS analysis involving compact and affordable MS analyzers might involve the identification and quantification of active pharmaceutical ingredients (APIs) and their degradation products or trace impurities, as reported for the analysis of a mixture of loratadine,[160] where impurities at low levels (0.1%) where identified with adequate sensitivity by a compact Advion MS analyzer. In another configuration, consisting of a SFC system coupled to an ACQUITY QDa, the quantitative determination of panthenol (provitamin B5) enantiomers in cosmetic formulations was achieved with a limit of quantification (LOQ) of 0.5 µg/mL, with full validation of the method in terms of the linearity, precision and accuracy.[161] In another contribution, the quantitative determination of degradation products and process-related impurities of daclatasvir in a pharmaceutical dosage form was performed through a UHPLC-PDA/QDa workflow after hydrolysis, oxidative, photolytic and thermal stress conditions.[162] In addition, the method was validated in terms of its specificity, precision, linearity, accuracy, LOD, LOQ and robustness according to ICH guidelines.

Compact benchtop MS systems are also suitable for high-throughput analysis or metabolomic assays. As an example, a workflow consisting of classical protein hydrolysis and derivatization with the fast separation of amino acids in plant materials and detection by UHPLC-QDa for high-throughput analysis was recently reported.[163] A targeted *in vitro* cytochrome P450 (CYP) metabolomic assay, focused on the separation of a mixture of 8 substrates and their CYP-specific metabolites, was also successfully performed by UHPLC-QDa,[164] with LOQ values between 2 and 100 ng/mL, and by SFC-QDa, with LOQ values ranging from 2 to 200 ng/mL.

Applications for biotherapeutics analysis are also feasible if peptides and proteins are of small to moderate sizes (up to a maximum of 6 kDa). In fact, identification is possible within this size range because the ESI ionization process generates multiply charged ions that bring the *m/z* values into the mass range of the simple quadrupole mass analyzer, therefore allowing their detection that would be otherwise hampered for the molecular ion alone. In this context, the latest contributions show the feasibility of UHPLC-QDa workflows for the analysis of peptides (angiotensin II, ~1 kDa) and small proteins (insulin, ~5 kDa), with detection and quantitation at the femtomole level.[154] In addition, the improved sensitivity and

specificity for identity and purity testing of intact insulin, gained through the addition of compact MS detection, was discussed by Zhang *et al.*[165] In this specific workflow consisting of LC-UV/QDa, the addition of MS detection also enabled the analysis of low abundance impurities.

Finally, the potential application of compact mass detectors in open access (OA) environments or in routine pharmaceutical quality control (QC) environments has already stimulated the imagination and curiosity of several research groups. In fact, after overcoming the factors limiting the widespread use of high-end MS instruments (high purchasing costs, expensive maintenance, and need for highly skilled operators for method optimization and subsequent data interpretation), the use of MS in OA or QC environments can be concretely considered a reality.

In a contribution by Gao *et al.*,[159] the suitability of compact mass detectors as potential OA LC–MS platforms in the drug discovery and early development space was discussed. The benefits of compact mass detectors were demonstrated in a wide variety of OA applications, such as standard small molecule analysis, reaction monitoring, purity assessment, high throughput screening (HTS) and QC-type analyses as well as in bioanalysis. Interestingly, mass data generated with the compact mass detector were comparable to conventional mass spectrometer data for a multitude of samples run in the OA environment.[159]

Similarly, D'Hondt *et al.*[166] investigated the possibility of implementing compact MS detectors in existing HPLC/UHPLC equipment and software platforms used in QC. A UHPLC-UV/MS equipment set-up was evaluated in relation to a traditional HPLC-UV set-up for the QC and impurity profiling of complex therapeutic peptides, namely the analysis of bleomycin sulfate, tyrothricin, vancomycin HCl and bacitracin peptide APIs. As expected, the UHPLC separation resulted in a higher resolution and a lower limit of detection as well as a significant reduction in the run time. Furthermore, the MS detector enabled the direct identification of impurities and components, even at low levels, without the need for reference standards.[166]

8. Process analytical technology

Process analytical technology (PAT) has been defined as a mechanism to design, analyze and control pharmaceutical manufacturing processes through timely measurements of critical quality attributes during processing.[167] Thanks to PAT, errors can be corrected while the process is in progress so that material losses can be avoided or strongly reduced during an industrial process. Applying this PAT approach has the ability to save the time and money required for product sampling and analysis and to produce quality and stable products in a shorter period of time.[168]

Many of the progresses in continuous manufacturing are closely related to the Food and Drug Administration (FDA) Pharmaceutical Quality for the 21st Century Initiative, which aims at promoting the modernization of pharmaceutical manufacturing thanks to the implementation of quality by design (QbD) and PAT to enable new manufacturing technologies.

Therefore, there have been many attempts in the last few years to integrate PAT and continuous manufacturing within pharmaceutical industries.[169] In many cases, spectroscopic-based tools such as ultraviolet-visible (UV-vis), near-infrared (NIR), Fourier transform infrared (FT-IR), and Raman spectroscopy have been successfully used for real time monitoring and process control,[170] including the monitoring of extraction and crystallization processes, the production of solid dosage forms, and the quantitation of active substances and impurities.[168] However, these approaches often lack specificity and sensitivity for several other PAT applications. For this reason, chromatography might be considered to be a useful strategy considering its excellent robustness, specificity and resolution. As recently reported, the implementation of on-line HPLC typically includes three steps: *(i)* a mechanism for automated sampling, *(ii)* sample pretreatment such as dilution, and finally *(iii) the* actual chromatographic and data analysis.[170]

On-line LC for process monitoring has been previously demonstrated in the pharmaceutical industry, but the instruments were often customized designs to support only one given process. Such an instrumental setup, where a traditional off-line HPLC has been transformed for on-line analysis, was recently proposed by Tiwari *et al.*[170] In this study, the authors have used a 2 way/6 port valve to facilitate the simultaneous automated sampling of product stream elution from a process column and fractionation. In the proposed methodology, no sample dilution was required. A fast HPLC separation was performed, and the versatility of the system has been demonstrated through the monitoring of two of the most common separations required during the production of mAbs, namely the separation of charge variants and aggregates. This study demonstrates that the proposed on-line HPLC configuration can be used for PAT applications in preparative chromatography to facilitate real-time decision-making. As a possible continuation of their work, the authors also mentioned that the proposed setup could also be integrated with UHPLC to further increase productivity (faster analysis times).

Despite this successful example a customized on-line LC system, such a technical solution often remains expensive to develop, and above all, its flexibility and portability remain clearly insufficient. Recently, commercial on-line LC instruments have been introduced (e.g., Waters PATROL UPLC Process Analysis System) offering an integrated and more robust PAT approach.[171] Patel *et al.* have recently highlighted the possibilities offered by such a

commercial system for on-line LC as a PAT tool for controlling mAb fermentation and purification processes through the continuous monitoring of biological processes and forced degradation studies. This work focused on ion exchange chromatography for the detection of charge variants. First, it is important to note that no significant differences were observed in the mAb charge heterogeneity profile with at-line and on-line sampling. The on-line method also had the ability to rapidly detect changes in protein quality over time. The robustness and versatility of the PAT methods were tested by sampling from two purification locations in a continuous mAb process, demonstrating that the distribution of acidic, main, and basic species percentages remains unchanged over a period of two weeks. Then, a forced degradation study showed an increase in acidic species and a decrease in basic species when sampled on-line over 7 days. According to the authors, implementation of on-line IEX also has the potential to be applied to biomanufacturing.[171]

Last but not least, a similar PAT instrument was also recently applied as an on-line tool for the crystallization of three active pharmaceutical ingredients (i.e., caffeine, acetylsalicylic acid and a patented drug from GSK). Indeed, concentration is an important process parameter in pharmaceutical crystallization processes, and the real-time multicomponent concentration was monitored in pharmaceutical crystallization[172] with PAT technology.

To date, the number of applications dealing with the implementation of on-line LC is still limited, but it will certainly rapidly grow due to its obvious benefits.

9. Automated tools for method development in chromatography

Method development in HPLC intends to obtain the optimal chromatographic operating conditions (i.e., types of mobile and stationary phases, temperature, gradient steepness, pH, ionic strength, etc.) resulting in the required separation of a mixture of compounds into its constituents within a reasonable analysis time.[173] The method development process is often tedious and time-consuming (up to several weeks of work). It requires knowledge and expertise from the analyst and still involves many trial and error processes. However, computer-assisted and automated method development tools have the potential to speed up this process. Systematic method development typically involves two phases, namely the scouting and optimization phases. Structure-retention databases are of interest to speed up the scouting phase and could potentially replace the initial exploratory experiments by prediction solely based on the structures of the molecules. Besides speeding up the method development process, systematic experimentation using state-of-the-art software packages also allow the Quality by Design (QbD) principles required in pharmaceutical laboratories to be met by providing a tool to improve the robustness of a chromatographic method eminently

important for a safe production process. In addition, computer-assisted method development reduces the solvent consumption by limiting the required number of experiments.

To find the most appropriate stationary phase and mobile phase conditions, it might be useful to initially perform a screening process (often known as “scouting”), as prior knowledge influences the choices.[164,174–176] Screening is typically performed on three or four selected columns possessing different chemistries and operating at three different mobile phase pH values in addition to using two different organic modifiers and performing a generic (short) gradient. Then, the number of resolved peaks, peak shapes and elution windows are usually compared to determine the best combination of stationary phase and mobile phase. This approach has been successfully applied for a mixture of eight probe substrates and 8 CYP-specific metabolites as well as for the separation of phytocannabinoids and *Cannabis sativa* extracts. [6,164]

Today, several commercial software programs and associated hardware are available for automating the mobile phase and column screening, including Nexera Method Scouting Solutions™ from Shimadzu, ChromSwordAuto™ and ChromSword Scout™ from Thermo-Fisher Scientific, AutoChrom™ from ACD labs and Fusion™ Method Development from Waters.[177,178] To utilize these software programs, certain hardware configurations (e.g., multichannel pumps and selection valves for columns) are required. The evaluation of multiple mobile and stationary phases in an automated fashion reduces the method development time and allows for efficient exploration of the separation parameters. As an example, using the Nexera Method Scouting Solutions software, up to 96 combinations of columns and mobile phase compositions can be screened in an automated way.[176] It is also worth mentioning that column kits dedicated for method screening/development are also available (e.g., Restek USLC Method Development Toolbox, Agilent Method Development Kits, Ascentis and Discovery Method Development Tool Kit from Sigma-Aldrich and Waters Method Development Kits). It is suggested that the initial screening be performed on short narrow-bore columns (e.g., 50 x 2.1 mm) to minimize the screening time and reduce solvent consumption.

For the optimization phase, several HPLC modeling computer programs have been developed in the last 25 years. The DryLab software was the first tool that predicts chromatograms under a much wider range of experimental conditions than would ever be possible to perform in a laboratory.[179] One can quickly and easily determine exactly how the separation would behave when a “virtual chromatographer” simultaneously varies multiple parameters. The main benefit of such software programs (i.e., DryLab, Osiris, ADC LC simulator and AutoChrom, ChromSword Developer and ChromSword Off-line) is that by using initial data generated from only 2–12 input experiments, the resolution and retention

times for hundreds (up to millions) of unique virtual conditions can be calculated. As an example, DryLab uses real data to create color-coded maps plotting critical resolution as a function of one, two or three method parameters. In addition to visualizing the interactions of these parameters, one can also predict chromatograms for changes in other method conditions, such as the column dimensions, flow rate, gradient elution, instrumental parameters, and many others.

In this contribution, we focus only on recent developments, and therefore, only the latest features and possibilities of such software programs are briefly discussed below.

The new 3-D resolution cube extends the previous 2-D retention models into the third dimension, providing a method operable design region (MODR) comprised of three variables in which the multifactorial variability for robust HPLC conditions is visualized (Figure 11 A).[179] In this way, the optimal conditions in a wide design space can be easily found. A special view can illustrate the 3-D regions that fulfil the predefined resolution criteria (for example, baseline separation of all peaks) (Figure 11 B). Another interesting new feature is the recently introduced column comparison module. This feature allows visual comparison of the parts of design spaces obtained with different columns, where the analytical target profile (ATP) for a selected critical resolution is fulfilled. The section of robust spaces can then easily be found by overlapping design spaces.[180] It is now also possible to perform virtual robustness testing without the need for additional time-consuming measurements.[181] The possibilities of 3-D resolution optimization have recently been shown by Rácz *et al*. The separation of amlodipine and 7 related impurities has been performed in an extended 3-D design space including the gradient time, ternary composition and mobile phase pH as method variables.[182] A similar approach was used for terazosin impurity profiling using MS-supported peak tracking. An old pharmacopeia method (requiring 90 min of analysis) has been re-worked, and finally, a 5 min long UHPLC separation has been proposed.[183] The RPLC column batch to batch repeatability was also studied by means of similarity in their 3-D resolution maps and applied for impurity profiling in the pharmaceutical industry.[184]

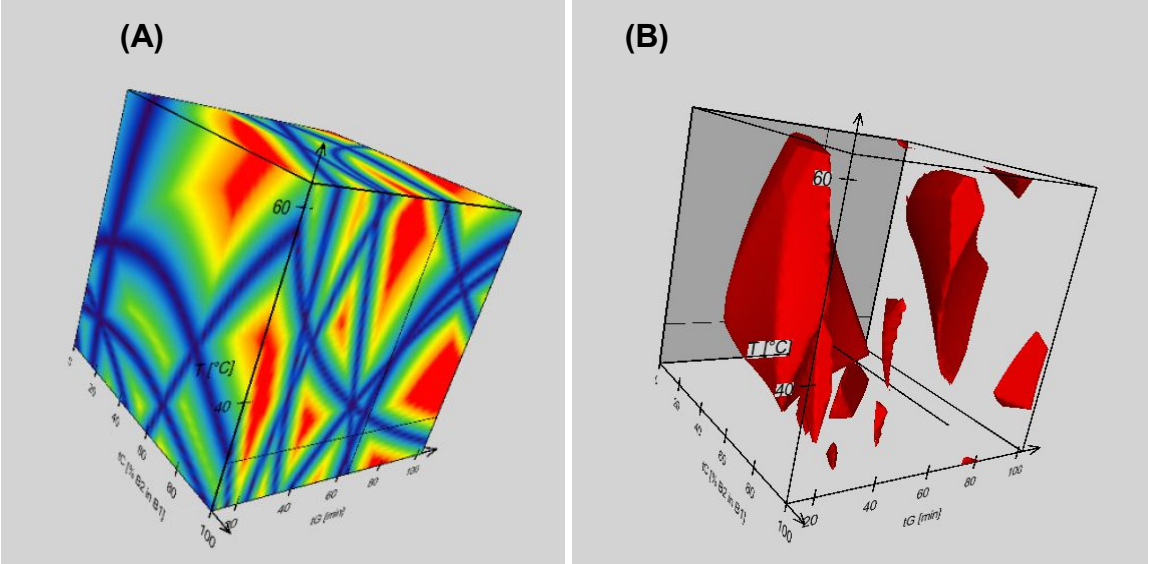

Figure 11. The illustration of a 3-D resolution cube. Baseline resolution regions are displayed in red; they are the method operable design region (MODR) visualized design space. Blue regions indicate method failure, where the critical resolution is equal to 0, which corresponds to peak overlaps (A). Baseline resolution regions are shown in red (B). Reprinted from Software-assisted method development in high performance liquid chromatography, Szabolcs Fekete, Imre Molnar (Ed), pp 30 (ref [179]). Copyright 2018, with permission from World Scientific.

Besides the three main model variables (typically gradient steepness, temperature, pH or ternary composition), the flow rate as well as the initial and final compositions of the mobile phase represent the investigated variables in a built-up model. The effects of these six variables can be calculated at three levels, corresponding to 3^6 = 729 variants in the selectivity. Virtual robustness testing has been successfully applied in practice for amlodipine impurity profiling on five different RPLC columns.[181] Kormány and co-workers reported in a following study an extended virtual optimization and robustness testing for multi-API products performed on 25 RPLC columns.[185] If method development is performed in a highly regulated environment, which is typically the case in the pharmaceutical industry, a great deal of documentation is required, and a comprehensive method development report can be generated in an automated way.

To have a method compatible with any column dimensions and HPLC or UHPLC instruments, the optimized methods can also be virtually transferred to other columns of different lengths, inner diameters and particle sizes as well as for various system gradient delays and extra-column volumes. For such a simulated method transfer, the initial data, acquired on a given column and system, have to be virtually changed, and the geometrical method transfer rules have to be considered. Kormány *et al.* reported on a successful computer-assisted method transfer between several column dimensions and various LC instruments for loratadine impurity profiling.[186] Retention modeling has been successfully applied for small solutes in various modes of chromatography (RPLC, NPLC and ion-pairing

RPLC) for a long time. Recent works proved the applicability of retention modeling for large molecules (biopharmaceuticals) in IEX, HIC and HILIC modes as well.[187] In contrast to small molecules, large molecules such as proteins show different retention mechanisms including the following: *(i)* an on/off mechanism retaining the macromolecules at the column inlet until at some point in the gradient they are desorbed and then move through the column without any further interaction; *(ii)* precipitation-redissolution, i.e., separation based on solubility instead of interaction with the stationary phase; and *(iii)* multipoint attachment to the surface of the stationary phase.[173,188,189]

While these mechanisms are fundamentally different from those observed with small molecules, the gradient separation of macromolecules can still be predicted from the linear solvent strength (LSS) theory or from slightly modified models. The reason is that in most cases, a relatively limited range of the method variables has to be studied because sufficient retention, recovery and peak shape can only be obtained in a limited design space. The other facilitation with large molecules is that generic conditions can be applied for different protein classes (e.g., cytokines, mAbs, and antibody drug conjugates (ADCs)). Indeed, the structures of the different proteins within a class are very similar; the amino acid sequences are very close, and the global conformations are similar. It is also clear that the variants, which have to be separated from the native protein and from each other, possess relatively small differences compared to the native protein (such as oxidation or deamidation of some amino acids or reduction of a disulfide bond). In a whole protein structure, those modifications are minor compared to the native amino acid sequence (e.g., modification of 2-5 amino acids from the total few hundred or thousand amino acids in the protein backbone).

From a modeling point of view, two modes of chromatography are often considered as problematic since accurate retention models are hardly derivable. One of these modes is HILIC. In HILIC, the main issue is the mixed retention mechanism due to co-existing hydrophilic partitioning, hydrogen bonding and possible electrostatic interactions between the solute, mobile phase and stationary phase. There are five accepted models developed and applied for HILIC separations (LSS, Neue-Kuss empirical, adsorption, mixed-mode and quadratic models). A recent study compared the accuracies of these different retention models for a wide range of analytes.[190] Gradient elution equations were developed for each model to approximate the integral for the case when no exact solution exists. For most compound classes, the adsorption model was found to provide the most robust performance. However, prediction accuracies depended on the analyte class and stationary phase. A design-of-experiment (DoE)-based model was also recently developed, which was able to describe the retention times of a mixture of pharmaceutical compounds in HILIC under various conditions.[191] Furthermore, a quantitative structure retention relationship (QSRR)

model was developed to predict the retention times for new solutes based only on their chemical structures. A compound classification, based on the concept of similarity, was applied prior to QSRR modeling. With the combination of QSRR and DoE approaches, a workflow has been proposed to facilitate HILIC method development. Other QSSR models were also developed to predict the retention times on five different HILIC phases to select the most suitable column.[192] To overcome the problem of HILIC retention modeling, Tyteca *et al.* proposed the use of the so-called predictive elution window shifting and stretching (PEWS^2) approach.[193] In this computer-assisted strategy, only approximate predictions of the retentions of the first and the last peaks in the chromatogram are required to conduct a well-targeted trial-and-error search, with the suggested search conditions uniformly covering the entire possible search and elution space. This strategy was used to optimize the separation of three representative pharmaceutical mixtures possessing diverse physicochemical properties. Another study showed that building models based on clustering the compounds and their similarity (retention factors) appeared to be an effective approach in minimizing the prediction errors in HILIC.[194] The concept of chromatographic similarity in QSRR could be implemented by localized modeling using a measure of similarity that adequately reflects solute retention. A recent review summarized the possibilities of chemometric-assisted method development in HILIC.[195] Some recent works focused on tryptic peptide retention modeling in HILIC for proteomic purposes.[117,196,197] Those models can help with peptide identification from protein digests and decrease the risk of false positive results.

The other critical mode of chromatography (from the method development point of view) is SFC due to the nature of the mobile phase (compressible and less viscous) and its mixed-mode retention mechanism. Further complications come from the fact that practical SFC mostly applies sub-critical conditions, and therefore, the mobile phase is often more liquid-like or possesses a hybrid state between supercritical fluid and liquid. Therefore, the temperature and pressure often work against each other and have a competitive effect on the retention. Significant changes are expected in retention when slightly changing the back-pressure, flow rate, mobile phase co-solvent proportion, additive concentration or mobile phase temperature. For all these reasons, retention in SFC is less controllable compared to LC, and retention modeling is hardly feasible. Recent works suggested different ways to perform SFC method development.[198] As example, a generic method development approach was proposed by De Klerck *et al.* to speed up the process of (UHP)SFC chiral separations of drugs.[199] Delahaye *et al.* reported the application of stationary phase-optimized selectivity predictions for isocratic SFC separations by demonstrating the applicability of this tool to predict the retention of solutes on different columns under isopycnic conditions.[200] Dispas *et al.* proposed the transfer of the whole design space (DS) when sending the method to a

receiving laboratory as being a useful tool to assist and speed up the commonly used geometrical transfer.[201]

Despite the huge potential of chromatographic modeling, computer-assisted and automated method development tools are still not widely used in the pharmaceutical industry. The reason is probably that most QC and routine laboratories apply previously developed older methods (suggested by Pharmacopeias and guidelines). Regulatory agencies and pharmacopeias should promote software-assisted method development, which makes studying the robustness, method transfer and method adjustment much more flexible and systematic as well as less time-consuming.

10. Chiral separations

Since many drugs are chiral, pharmaceutical companies are putting increasing efforts toward the development of improved and well-controlled enantioselective manufacturing processes. Stereoisomers of a chiral drug can indeed differ in potency, toxicity, and behavior (pharmacodynamics) within biological systems as well as in terms of their absorption, distribution, metabolism, and excretion (pharmacokinetics). Inter-conversion of stereoisomers is also possible for some chiral molecules due to their configurational instability around the chiral centers. Racemization or epimerization can occur at any time during the pharmaceutical and pharmacological time scales (development, manufacturing, storage, and time spent in the body under physiological conditions).

Continued discoveries of significant differences in the effectiveness and toxicity of individual enantiomers in biological systems has maintained the importance of chiral separations and motivated the development of new chiral stationary phases (CSPs). More than 110 CSPs have been introduced during the last 15 years, but there are now a few preferred ones, which satisfy the majority of chiral separation needs in the pharmaceutical industry. Cellulose and amylose phases are still very popular, while the macrocyclic glycopeptide applications have significantly expanded and taken on a complimentary role. Cyclodextrin derivatives and π-complex Pirkle-type CSPs (e.g., Whelk-01) have fulfilled the remaining needs. A new generation of immobilized polysaccharide CSPs was also introduced a few years ago. One of the most successful ones is Chiralpak IC, which is a covalently bonded tris(3,5-dichlorophenylcarbamate) derivative of cellulose. This phase had never been made available as a coated version due to its high solubility in organic solvents. As a bonded phase, it has demonstrated good selectivity for those analytes previously not resolved or poorly resolved by the other immobilized cellulose and amylose derivatives. Other coated derivatives such as the methylchlorophenyl carbamate of cellulose and of amylose are also available today.

These phases were originally developed on 20 µm silica particles for preparative applications only, but they are now available with smaller particle sizes for analytical applications.

In the last decade, the attention of column developers has moved from the research of novel CSPs to the preparation of new versions of already known CSPs prepared on high performance silica particles, such as sub-2 µm FPPs and sub-3 µm SPPs.[202] Based on the needs from pharmaceutical industries, e.g., for the screening of large libraries of chiral molecules or for high-throughput analysis, providers have developed columns for faster and more efficient chiral separations, which could not be achieved earlier on conventional CSPs. Pirkle-type (often referred to as brush-type) CSPs were the first ones employed for the preparation of sub-2 µm chiral particles.[203] The first Pirkle-type UHPLC CSP was prepared in 2010 (FPPs of 1.9 µm with a DACH-DNB selector). The kinetic performance of columns packed with this CSP was compared to those of columns packed with 4.3 and 2.6 µm particles. The sub-2 µm column displayed both a significant efficiency gain and reduced analysis times, while maintaining comparable selectivity and improving the resolution. Barhate *et al.* functionalized narrow particle size distribution particles (1.9 µm) with macrocyclic glycopeptide selectors.[204] Sub-minute separations of amino acids, β-blockers and heterocyclic compounds of pharmaceutical interest were demonstrated. A new synthetic process was recently reported to produce the zwitterionic version of teicoplanin sub-2 µm CSP and applied to separate the enantiomers of amino acid derivatives of polar acidic compounds. It also showed large selectivity for organic/inorganic ions.[205,206] To further speed up chiral separation, Gasparrini *et al*. combined the use of a short column (1 cm length) and high flow rates (8.0 mL/min). Figure 12 shows an example of such an ultra-fast approach. For the sake of completeness, some other chiral UHPLC fast separations are reported in a recent review.[207]

Similarly to other modes of chromatography, besides sub-2 µm fully porous particles, sub-3 µm SPPs have also been applied for chiral separations. A cinchona alkaloid-based CSP was prepared on 2.7 µm SPPs in 2011. Chankvetadze *et al.* developed different polysaccharide-based CSPs by coating the polymeric selector on 2.6 µm SPPs. Comparison with FPPs having a similar content of chiral selector showed a higher efficiency at higher flow rates for the SPP *vs.* FPP.[208,209] Separations were carried out in the 15–30 s time scale. Hydroxypropyl-β-cyclodextrin, cyclofructan-6 based selectors, teicoplanin, and vancomycin were also recently bonded on 2.7 µm SPPs.[210–213] It was found that chiral SPPs outperformed their FPPs counterparts in all working conditions ranging from the normal phase to the polar organic mode and HILIC. Some authors reported unexpected results when comparing the behavior of brush-type Whelk-O1 CSPs made on 2.6 µm SPPs with those of the same selector bonded on both 1.8 and 2.5 µm FPPs.[214]

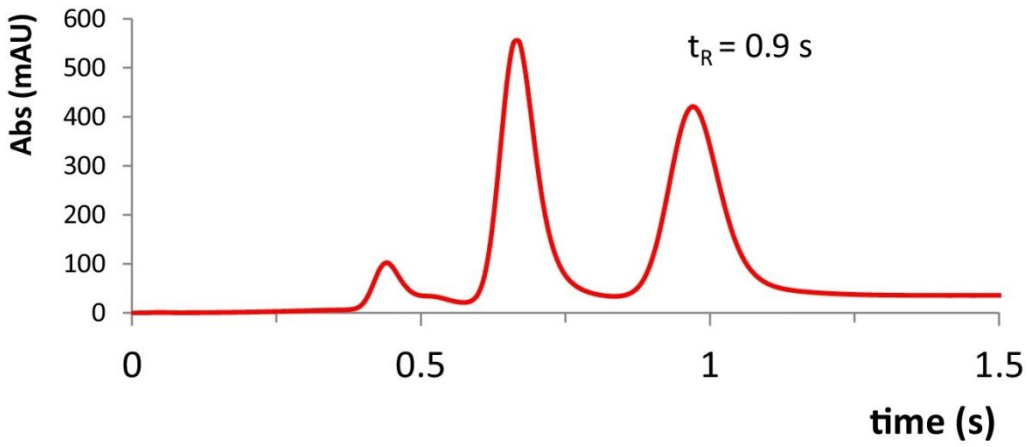

Figure 12. Example of ultrafast enantioseparation (enantiomers of trans-stilbene oxide) thanks to the use of a short column and high flow rate. A 10 × 3.0 mm column was operated at 8 mL/min. Reprinted from J. Chromatogr. A, Vol. 1466, Ismail, O. H.; Pasti, L.; Ciogli, A.; Villani, C.; Kocergin, J.; Anderson, S.; Gasparrini, F.; Cavazzini, A.; Catani, M. Pirkle-type chiral stationary phase on core–shell and fully porous particles: Are superficially porous particles always the better choice toward ultrafast high-performance enantioseparations?, pp 96–104 (ref [214]). Copyright 2016, with permission from Elsevier.

Chiral separations are still one of the workhorses for SFC since the analysis and production of enantiomerically pure compounds is still a major topic of interest when active pharmaceutical ingredients are concerned. There are a lot of improvements that were brought to chiral SFC analysis thanks to the use of modern systems and columns packed with small particles.[207,215] Using such a combination of an instrument with a low extra-column volume and the high kinetic performance of chiral columns packed with sub-2 μm particles, chiral separations in less than 10 s have been demonstrated[216] on a short immobilized polysaccharide stationary phase. However, to achieve such performance, care should be taken to have fully optimized instrumentation. Indeed, sub-minute chiral separations can be easily achieved with modern SFC instruments at high pressures of up to 580 bar and columns packed with small particles. Indeed, the high flow rates required for ultra-fast separations may result in a high extra column pressure drop in the tubing, causing a turbulent flow and some uncertainty in the true column inlet, outlet, and average pressure/density.[217] An alternative solution to achieve high throughput separations is to perform multicolumn parallel screening and multiple injections in a single experiment run (MISER). This approach has been successfully employed to increase throughput when working with a large number of samples.[218] It was demonstrated that the plate analysis time (time required for the enantiopurity analysis of 96 samples) of less than 34 min was achievable in the best cases. However, this approach remains limited by the speed of the autosamplers. Besides the speed of analysis, there is also still some interest in further improving the enantioselectivity and overall resolution, as an example, by using much higher additive concentrations in SFC (up to 10% 2-propylamine)[219] or by better understanding the enantiorecognition phenomenon[220] thanks to a recent comparison of normal phase LC and

SFC on 171 achiral probes and 97 racemates. Contrary to popular belief, SFC mobile phases are often worse than liquid mobile phases when applied for enantiorecognition.

Thanks to the recent advances in chiral chromatography, much faster separations can be performed today compared to earlier conventional separations. Therefore, chiral separations can now be used in 2D-LC. A recent study demonstrated that excellent selectivity, peak shape, and repeatability could be achieved by combining achiral and chiral narrow-bore columns packed with highly efficient chiral selectors (sub-2 μm FPP and 2.7 μm SPP) in the second dimension, together with the use of 0.1% phosphoric acid/acetonitrile eluents in both dimensions.[221] Multiple achiral × chiral and chiral × chiral 2D-LC (single and multiple heart-cutting, high-resolution sampling, and comprehensive) using ultrafast chiral chromatography in the second dimension were also successfully applied for the separation and analysis of complex mixtures of closely related pharmaceuticals and synthetic intermediates (including chiral and achiral drugs and metabolites, constitutional isomers, stereoisomers, and organohalogenated species). Figure 13 shows an example of a comprehensive chiral × chiral 2D-LC method for the complete resolution of isomers of a synthetic intermediate.

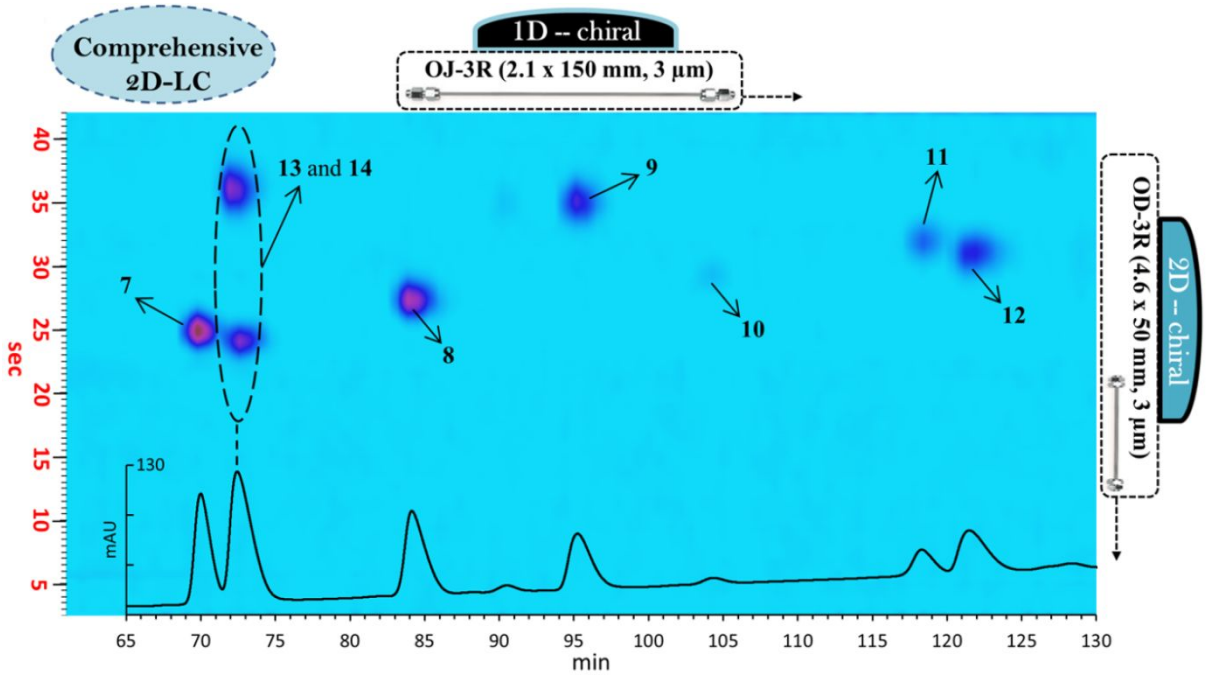

Figure 13. Comprehensive chiral × chiral 2D-LC method for the complete resolution of isomers of a synthetic intermediate. Conditions, first dimension (chiral): column, OJ-3R column (2.1 mm × 150 mm, 3 μm); flow rate, 0.05 mL/min; mobile phase, eluent A, 0.1% H_3PO_4 in H_2O and eluent B, ACN/MeOH (70:30, v/v %); step gradient, hold 30% B for 20 min; 20−120 min, 35% B; 120−120.8 min, 30% B; 120.8−160 min, 30% B; and 40 μL loops. Conditions, second dimension (chiral): column, Chiralcel OD-3R (4.6 mm × 50 mm, 3 μm); flow rate, 3.0 mL/min; and isocratic mobile phase, 0.1% H_3PO_4 in H_2O/ACN (60:40). Reproduced from Barhate, C. L.; Regalado, E. L.; Contrella, N. D.; Lee, J.; Jo, J.; Makarov, A. A.; Armstrong, D. W.; Welch, C. J. Ultrafast Chiral Chromatography as the Second Dimension in Two-Dimensional Liquid Chromatography Experiments, Anal. Chem. 2017, 89 (6), 3545–3553 (ref [221]). Copyright 2017 American Chemical Society.

11. Genotoxic impurities

Potential genotoxic impurities (PGIs) and genotoxic impurities (GIs)/mutagenic impurities (MIs) can be found in drug substances and pharmaceutical products, and include compounds resulting from chemical synthesis or subsequent degradation that are DNA reactive (mutagenic) and potentially able to cause DNA damage and/or mutations. Identification and control of PGIs/GIs/MIs are therefore required to limit potential human carcinogenic risk associated with their exposure. To answer the need for consistency among already existing guidelines on PGIs/GIs/MIs,[222–227] the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) elaborated specific guidelines for the determination, categorization, qualification, and control of DNA reactive (mutagenic) impurities in medicinal products (guideline ICH M7/R1).[228] According to the threshold of toxicological concern (TTC) concept, an acceptable lifetime exposure to mutagenic impurities is defined as a daily intake corresponding to 1.5 μg, meaning that for a dosage of 1 g of active pharmaceutical ingredient (API), any impurity must be less than 1.5 ppm (1.5 μg). However, even a lower level might be required for a small cohort of highly potent genotoxic compounds, including aflatoxin-like, N-nitroso, and alkyl-azoxy compounds. For the sake of comparison, acceptable concentrations of general (non-mutagenic) pharmaceutical impurities are at or below 500 ppm level (corresponding to 0.05% of API).

In the process of identification and control, impurities are first classified with respect to their mutagenic and carcinogenic potential. Known mutagens (Class 1), known mutagens with unknown carcinogenic potential (Class 2) and alerting structures for which no mutagenicity data are available (Class 3) are controlled at or below compound-specific acceptable limits or appropriate TTCs. Conversely, alerting structures that have been tested and are non-mutagenic (Class 4) and impurities with no structural alerts or alerting structures with sufficient data to demonstrate the lack of mutagenicity or carcinogenicity (Class 5) are treated as non-mutagenic impurities.[228] Once classified, a qualification strategy is applied to define the genotoxic potential of the impurity or to establish permitted specification limits for the impurity in the drug product. Based on the allowable daily intake (ADI) and TTC concept, the final goal is to establish and assess the acceptable limits of the impurity in the API, which generally consists of concentrations at the ppm level (namely μg/g) but that may well fall between 10 and 1000 ppb (10 ng/g and 1 μg/g, respectively). Achieving such a tiny level of detection and quantitation is extremely challenging since trace levels of genotoxic impurities might be present in complex matrices constituted by higher levels of the API and other impurities. To meet the official requirements, chromatographic approaches coupled to MS represent the cutting-edge analytical solutions because of their ability to provide high sensitivity and selectivity. In this context, several practical charts have been proposed to

discover the most suitable analytical method.[229–231] Based on the volatility of the PGIs/GIs/MIs, the selection of its analytical technique can lead to either gas chromatography (GC) or liquid chromatography (LC) analysis, whereas the choice of the detector is defined based on the required limits the impurity must meet while avoiding matrix effects.

For volatile PGIs/GIs/MIs, GC with flame ionization detection (FID) is generally applied as a first choice. Then, if the desired sensitivity is not obtained and the method cannot be finalized and validated, either other detectors are evaluated or the volatility and stability of the PGIs/GIs/MIs is improved through derivatization or extraction approaches to minimize the matrix effects. 2D-GC approaches can be applied as a last resort when all of the above-mentioned techniques were unsuccessful in terms of selectivity.[229,230]

As an example, a sensitive and reliable GC–MS method was developed, optimized and validated for the simultaneous determination of 4 closely related GIs, namely 3-chloro-1-propanol (CHP), 1,3-dichloropropane (DCP), 3-chloropropylacetate (CPA) and chloropropyl hydroxypropyl ether (CHE), contained in the drug fudosteine.[232] Analytes were extracted in dichloromethane and monitored by gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) in the selective ion monitoring (SIM) mode. The LODs and LOQs established for CHP, DCP, CPA and CHE were in the range of 50-80 ppb (0.05-0.08 µg/mL) and 100-170 ppb (0.10–0.17 µg/mL), respectively. Satisfactory separation, detection, and quantitation were achieved, and the method was assessed by evaluating the specificity, precision, sensitivity, linearity and accuracy.[232]

For non-volatile PGIs/GIs, RPLC is generally applied as the first choice coupled to either UV or MS detection depending on the chromophoric properties of the impurity. Then, other modes of chromatography might be selected for polar or ionizable compounds, namely HILIC and mixed-mode chromatography (MMC). Finally, 2D-LC(-MS) can be helpful when all of the other approaches do not meet the required selectivity and sensitivity.[229,230]

A comprehensive example of method development for PGI identification and characterization was recently published by Huang *et al.*[233] In this contribution, an unknown degradation product (impurity I) was detected during the stress testing of linagliptin, and it was subsequently isolated, identified, and structurally characterized by orthogonal analytical techniques (i.e., MS, MS/MS, 1D and 2D NMR spectroscopy, and IR spectroscopy). The degradation product (impurity I) and another process-related impurity (impurity II) of linagliptin were found to contain the structural alerts of N-acylated aminoaryl and alkyl halide, respectively, which are both PGI substances. Based on the TTC, the permitted level of these PGIs in linagliptin was 300 ppm. Therefore, an RPLC-UV method was purposely developed

for the simultaneous determination of these two PGIs by reaching LODs of 20 and 10 ppm and LOQs of 60 and 30 ppm for impurity I and impurity II, respectively.[232]

In another contribution, Dousa *et al.* reported the HILIC-MS determination of GIs of 2-chloro-N-(2-chloroethyl)ethanamine in the vortioxetine manufacturing process.[234] QDa mass detection operated in the positive ESI mode with the SIM acquisition mode was used for the quantitation of GIs at the 75 ppm level with respect to vortioxetine. The use of selective detection techniques such as LC-MS significantly increased the selectivity and sensitivity in comparison to UV detection. Notably, the GI was separated and quantified in its native form without any derivatization step or additional sample preparation. Finally, the method was developed and validated by meeting the requirements of regulatory agencies.

As a last example, the beneficial value of the derivatization process in enhancing the selectivity and the sensitivity of GIs during their identification and control was clearly highlighted for the analysis of the known GI hydrazine in pharmaceutical materials.[235] As reported by the authors, due to its physical and chemical properties (non-chromophoric, low molecular weight, high polarity and volatility), hydrazine is a challenging GI to analyze, and its identification in pharmaceutical samples suffers from important matrix effects. However, the proposed derivatization process of hydrazine, with 2-hydroxy-1-naphthalaldehyde (HNA) as a derivatization reagent, allowed the formation of a hydrazone product that streamlined the identification of the GI and its monitoring by RPLC-UV. In fact, thanks to the derivatization, several benefits were obtained, including the following: *(i)* an increased sensitivity by UV-vis detection (by the addition of chromophores to hydrazine); *(ii)* the elimination of the matrix effects (lambda max of hydrazone well-shifted away from the absorption wavelengths of the API); and (iii) accurate quantitation of trace levels of the GI (as a result of the increased resolution of the derivative product from the API and its related impurities). Finally, the method was validated and applied as a generic method to determine hydrazine for pharmaceutical process control and drug material release by meeting the expected detection limit (0.25 ppm).[235]

12. Cleaning validation

Documented equipment cleaning is required to establish the cleanliness of manufacturing equipment (production line) before its subsequent release for use in the production of intermediates and active pharmaceutical ingredients (APIs).[236] Non-dedicated equipment should be cleaned at product change to prevent cross-contamination for subsequent batches manufactured on the same equipment.[237] Cleaning procedures should contain sufficient details to enable operators to clean each type of equipment in a reproducible and effective manner, and these procedures should include a complete description of the methods and

materials, including the dilution of the cleaning agents used to clean the equipment. From an analytical point of view, cleaning verification/validation requires the development of validated methods, which are able to determine either *(i)* the residual APIs from the previous batch or *(ii)* the residual detergents, which are used to remove the drug product residues from the manufacturing equipment.

The basic principle of equipment cleaning is that the patient should not take more than 0.1% of the standard therapeutic dose (effective dose) of a previously manufactured product. Therefore, the maximum allowable carry-over (MAC or MACO) depends on the minimal daily dose (active weight) of the previous product, the smallest batch size of the subsequent product and the maximum daily dose (product weight) of the following product. An additional criterion is the 10 ppm limit, which suggests that not more than 10 ppm of the previously manufactured product is allowed to appear in the subsequent product. If the MAC value becomes greater than 10 ppm, then the 10 ppm criterion needs to be considered. For detergents, as many of them are considered to be slightly or moderately toxic based on their LD_{50} data, their carry-over is acceptable up to a 100 ppm limit. The determination of both the residual API and detergent requires very sensitive methods. Therefore, the assessment of residual APIs and detergents at the end of the equipment cleaning process is challenging because the analytical method has to address the limits of quantitation at trace levels (e.g., ppm) of analytes that often lack chromophores.

Analytical methods used to determine the residuals of APIs should be specific for the substance or class of substances to be assayed and be validated prior to cleaning validation. HPLC–MS and UHPLC-MS techniques applied in pharmaceutical cleaning verification have the advantages of improved sensitivity, selectivity and general applicability, even for UV-inactive compounds. However, these techniques are quite expensive and still not widespread in cleaning control analysis. The current trend seems to develop methods not only for a given API but in a more generic way. The concept of applying a generic method for several API residues for a product line was found to be feasible and practical if the structures and properties of the compounds to be determined are similar. For such a purpose, a generic UHPLC-UV method has been reported for the fast determination (analysis time of 2.5 minutes) of various steroid residues in support of cleaning validation. Another study described the development of a single 10 minute gradient HPLC/UV method for cleaning verification applications at 0.2 to 10 µg/mL levels for many APIs.[238] Additional sensitivity enhancement can be accomplished by large volume injections, sample enrichment and the use of long-path UV flow-cells. The general adoption of generic platform technologies can greatly enhance analytical laboratory productivity by simplifying lab operation and reducing method development and validation efforts. This standard method is readily adaptable to swabbing or rinse solutions.

For detergents containing chromophore groups, the HPLC-UV method is suitable for their detection and quantitation in most cases. If a chromophore is absent, other techniques such as ion chromatography with conductivity detection or charged aerosol detection can be used.[239]

The most critical parameter of the analytical methods applied for cleaning validation processes is the correct sampling and related solute recovery. The surface of the equipment-line consists of mostly (> 95%) stainless steel, but there are other critical surfaces, which are made of Plexiglas, polytetrafluorethylene, silicone (gaskets) and textiles. These specific surfaces are hard to clean, and thus, it is necessary to sample these areas during the cleaning verification/validation process (as they represent the “worst case” places of the equipment). During analytical method development and validation, the solute recovery from such surfaces needs to be checked. Sampling can be done either by swabbing (wiping) or rinsing, depending on the accessibility of the equipment part. Typically, model coupons (made of 10 x 10 cm stainless steel or other representative materials) are used for recovery studies. The API or detergent is added (spiked) in a known concentration onto the surface and then wiped using a wetted swab. The content is then extracted with an appropriate solvent. The parameters affecting the recovery of APIs from the surfaces of stainless steel coupons have been recently studied, including the API level, spiking procedure, API/excipient ratio, analyst-to-analyst variability, inter-day variability and cleaning procedure of the coupons.[237] The lack of a well-defined procedure that consistently cleaned the coupon surfaces was identified as the major contributor to low and variable recoveries. The assessment of acidic, basic and oxidant washes as well as the order of treatment, showed that a base-water-acid-water-oxidizer-water wash procedure resulted in a consistent and accurate spiked recovery (> 90%) as well as reproducible results. An interesting feature of such a modeling of API recovery was also pointed out, namely that the surface of the coupon may be modified during its use due to the deposition of a thin film of material on the surface, which may result in altering the oxidation states of the metals on the surface. Therefore, a time-dependent decrease in recovery is expected due to binding between the metals and the analyte of interest. Indeed, the repeatability of sampling decreased with an increasing number of replicates, and therefore, the regeneration of coupon surfaces seems to be mandatory. Another study showed that a good correlation could be found between the recoveries of solutes and their retention factors measured in RPLC. More hydrophobic solutes showed worse recoveries in most cases. Silicone rubber surfaces were found to be the most critical surfaces during the sampling process. It is recommended to apply these silicone gaskets as dedicated parts of the equipment.

13. Greening LC

In recent years, greening the methods for the analysis of drugs during the pharmaceutical drug development process has been receiving great attention, with the aim to eliminate or minimize the amount of organic solvents consumed daily worldwide. As example, a standard LC column (250 x 4.6 mm, 5 µm) used at a mobile phase flow rate of 1 mL/min generates approximately 1.5 L of liquid waste daily,[240] corresponding to approximately 500 L of waste per year. Although this volume of waste looks small compared to the amount generated by a large industrial company, some big companies use hundreds of LC systems in their research laboratories and in process control laboratories, resulting in thousands of liters of toxic waste produced every day.[241] The environmental impact of solvents consumed during chromatographic measurements should thus not be neglected since they contribute to a negative environmental impact.[242] The concept of green analytical chemistry has recently emerged, and the 12 principles originally suggested by Anastas[243] are now often considered when developing new analytical methods.[244,245] There are various solutions to make LC more eco-friendly, but unfortunately, they are not always considered during the method development process. Among them, we can cite the following: *(i)* reduction of organic solvent consumption and organic waste production; *(ii)* replacement of toxic organic solvents; and *(iii)* miniaturization of chromatographic techniques.[240,241,246] These three strategies and their applications in the pharmaceutical industry will be described hereafter.

First, the reduction of solvent consumption may be achieved by increasing the chromatographic productivity. This is, for example, achieved by using shorter and narrower columns packed with sub-2 µm fully porous particles or sub-3 µm superficially porous particles. This strategy increases the throughput and consequently reduces the waste. However, such approaches cannot be easily implemented on regular HPLC systems, and UHPLC instruments compatible with pressures up to 1000-1500 bar have to be preferentially employed, as previously described. UHPLC is increasingly widely used in the pharmaceutical industry today, not only to increase analysis throughput but also to reduce solvent consumption. As an example, between a HPLC column of 150 x 4.6 mm, 5 µm and a UHPLC column of 50 x 2.1 mm, 1.7 µm producing the same plate count, the solvent consumption can be reduced by approximately 14-fold when applying geometrical transfer rules. Therefore, using short narrow-bore columns packed with sub-2 µm particles in combination with UHPLC is a valuable approach for greening LC. Using elevated temperature is also another way to reduce solvent consumption. Indeed, the polarity of water is reduced at higher temperature, and therefore, it becomes possible to replace a significant part of the organic solvent in the mobile phase with water.[247] It has even been demonstrated that at sufficiently high temperature (150–200 °C), only pure water can be used as a mobile phase. This strategy

has been described as “superheated water chromatography” or “subcritical water chromatography” and is a promising eco-friendly substitute for regular RPLC.[248] However, this approach is not very widespread in the pharmaceutical industry simply because silica-based stationary phases are not sufficiently stable at temperatures beyond 100 °C and because there is always a risk of on-column degradation, which could be very critical when performing the impurity profiling of drugs.

The next strategy for greening LC is to use greener mobile phase components. In particular, acetonitrile and methanol have been replaced by acetone or ethanol, which can both be considered to be more environmentally friendly mobile phases. Ethanol is particularly desirable since it has close properties to methanol but is less volatile, less toxic and has lower disposal costs.[249,250] However, there are three chief barriers to the replacement of acetonitrile with ethanol, namely, the high viscosity, substantially different chromatographic selectivity, and restrictions in trading. To avoid this latest issue, some people from the pharmaceutical industry have suggested performing chromatography with distilled alcohol spirits (i.e., cachaça, rum, vodka, aguardiente and grain alcohol). It is indeed available at a relatively inexpensive price from local markets in virtually all regions of the world, offering the possibility of local sustainable production of a green nontoxic eluent for HPLC. This strategy was named “cocktail chromatography” and offers, in many cases, excellent analytical performance.[251] However, such an approach cannot be applied in the pharmaceutical industry. Acetone is another alternative, but it is scarcely used as a mobile phase component since it is a strong UV absorber up to 340 nm, which makes it impractical when UV detection is used.[245] Replacing organic solvents with carbon dioxide under supercritical conditions is another powerful approach to make LC greener,[252] since it is characterized by non-toxicity, non-flammability, low disposal costs and above all limited environmental impact (see Section 4 on SFC). In HILIC separations, the addition of CO_2 to the conventional mobile phases makes the replacement of acetonitrile with methanol or ethanol possible, resulting in greener separations. This strategy has been recently applied for the analysis of nucleosides/nucleotides,[253] amino acids,[254] drugs and natural products.[254]

The last strategy to minimize organic solvent consumption is to play with the column-related parameters. Indeed, when reducing the column internal diameter, the flow rate has to be scaled down by the square of the column diameter without affecting the analysis time as well as peak width and provided that the extra-column volume remains acceptable. Moving from the classical 4.6 mm to 3 mm I.D. leads to a 57% reduction in the volume of solvent and waste. Today, the use of narrow-bore (2.1 mm I.D.) columns is becoming increasingly popular in the pharmaceutical industry due to the significant progress in chromatographic instrumentation over the last few years (UHPLC instruments with much lower extra-column

volumes). Micro-bore (1 mm I.D.) and capillary (0.3 mm I.D.) LC columns constitute the ultimate approach in this direction but require specific instruments,[246] which are not yet widespread in the pharmaceutical industry.

14. What are the latest advances in GC?

Despite Gas Chromatography (GC) predating HPLC, it still remains a lesser used technique in pharmaceutical analysis owing to the insufficient volatility and thermal stability of the majority of APIs. Nevertheless, GC and GC–MS remain essential tools in qualitative and quantitative pharmaceutical analysis, including: residual solvents in APIs and pharmaceutical products and ingredients; volatile organic compounds (VOCs) such as toxic and genotoxic impurities and degradation products (see Section 11); extractables and leachables; and also the analysis of raw materials, (e.g. solvents and reagents) and starting materials/synthetic building blocks (e.g. impurity profiling and characterization). When pharmaceutical analytes are amenable to GC, it is often the technique of choice as it has multiple advantages over HPLC, owing to it high efficiency and resolving power, simple operation, and a universal-like response and wide dynamic range of the flame ionisation detector (FID).

Fast GC using narrow-bore/micro-bore columns and fast temperature programming rates was developed to decrease analysis times. This was followed by ultra-fast GC (UFGC) using resistively heated temperature programming with ballistic heating rates, which lead to faster analysis times (2-20 times), thus reducing power and also carrier and detector gas consumption.[255,256] Fast GC/Ultra-Fast GC has been successful applied in pharmaceutical applications. However, despite these advantages, the uptake of these technologies in the pharmaceutical industry was limited owing to perceived poor usability, fragility/poor robustness, and higher costs. Recent enhanced commercial instruments (e.g. Agilent Intuvo™) have renewed interest in UFGC owing to their reduced footprint and improved workflows and usability via features such as simple or ferrule free connections, ease of changing guard and analytical columns, improved serviceability/reduced maintenance, and improved user interfaces etc.[256,257]

In recent years, there has been several workflow-based innovations related to increasing productivity *via* decreasing overall analysis times using existing technology, but with alternative methods and ways of working. Scientists from most Pharmaceutical R&D groups have developed generic methods for static headspace GC for the determination of residual solvents in APIs and drug products.[258] Several R&D groups have also developed and implemented open access GC and GC-MS systems using generic methods, and also automated method development screening systems with columns offering different

selectivity, in dual column or parallel column formats. These include systems for impurity profiling and chiral analysis of volatile raw and starting materials and chemical intermediates. Additionally, various generic GC-FID relative response factor (RRF) methods have been developed and employed with either direct injection or headspace sample introduction. Here, pre-determined RRFs against an internal standard (e.g. Decane) were determined and utilised to quantify a wide range of residual solvents of interest. These approaches based on lean sigma principles significantly improved the laboratory efficiency and are greener, as they significantly reduce the use of sample solvents compared to external standard methods. They have also been routinely employed in regulated environments (e.g. GMP).[259–261]

Several GC chromatographic modelling and method translator software are commercially or freely available. One such on-line modelling program called Pro-EZGC™ simulates separations using database information containing retention models of 100s of common volatile organic solvents and reagents on multiple columns, and provides GC conditions and column selection for analysis within *ca.* 5-10 minutes. Many method translators facilitate rapid conversion of conventional capillary GC methods to fast or ultra-fast conditions, or the evaluation of a change of carrier (e.g. He to H_2) and propose operating conditions whilst maintaining chromatographic resolution, or it can propose higher speed separations with a reduction in peak capacity and chromatography resolution. In the pharmaceutical industrial environment, these tools are very helpful and lead to significant time savings in method development and optimisation.

Multidimensional GC has had continued development. Previously, although it was recognised as a powerful technique, it was seen as an academic or research only tool. Other users often shied away from the technology, owing to the perceived high costs and difficultly to implement routinely in industrial laboratories. However, thanks to more affordable and flexible, modular hardware, along with simpler workflows, data processing and databases, it is now starting to be adopted in several industries by high-throughput laboratories and for comprehensive screening. In particular, multidimensional GC with multiple column and detection (e.g. dual detection MS and FID) has been shown to be a powerful technique in regulated areas of the fragrances and flavours industries.[262–264] Therefore, multidimensional GC is also transcending into pharmaceutical analysis, but one can expect less demand and utilisation owing to lower numbers of volatile analytes and the lower complexity of pharmaceutical samples. Trace analysis of Mutagenic impurities is one area where 2D-GC has been successfully applied owing to the improved selectivity (see section 11).[229,230,264] Metabolomics is another area where it has been utilised to support drug discovery and development. Several groups are developing new technologies and strategies for the

definition of the composition of volatile and non-volatile biologically active secondary metabolites of plants and plant products of interest in the pharmaceutical sectors.[265–267]

Recent advances in column technology have focused on improved usability with newer commercial stationary phases demonstrating higher inertness, lower bleed and higher maximum operating temperatures leading to reduced maintenance and conditioning times. These types of phases based on advances in polymer development and inert/deactivated surfaces have improved usability and reliability in routine GC operations and may further extend the interest in fast GC, specifically if there are high temperature stable and inert stationary phases available in UFGC format. The development and introduction of novel stationary phases is also needed for multidimensional and will further increase the use in the pharmaceutical industry as well as other industries.

Ionic liquid (IL) phases have unique selectivity and chromatographic properties, and have been a recent addition to the range of commercial available stationary phases and offer opportunities for extending the selectivity range and temperature-operating range compared to conventional columns.[268] Armstrong *et al.* have developed fully water-compatible ionic-liquid IL-based capillary GC columns based on derivatives of phosphonium or imidazolium cations combined with the trifluoromethanesulphonate anion. These IL-derivatives are stable to water and thus it be used as injection solvent, and is thus advantageous for water-based samples which often require extensive sample preparation procedures when using conventional columns. These commercially available water-compatible IL columns have been successfully applied in a number of industries for direct qualitative and quantitative analysis of aqueous samples. Armstrong *et al.* successfully developed sensitive IL-based capillary GC methods using thermal conductivity detection (TCD) or barrier ion discharge detector (BID) to determine the water content in liquid samples in pharmaceutical analysis.[269] These methods provides a suitable and more sensitive, but also more expensive alternative to existing Karl Fischer methodologies (e.g. when having interferences or side reactions, instability, insufficient solubility or limited sample quantities or when lower detection limits are needed). Further long term and wider use will demonstrate if these phases are sufficiently stable and robust, which has been questioned by some scientists and suggested the inertness and possibly the range of operative temperature needs to be extended when water is the main sample solvent.[270]

Recently, innovations in GC detector technology have included Vacuum Ultraviolet (VUV) and FTIR.[271] GC-VUV spectroscopy is claimed to be an easy to use universal detection that provides qualitative spectral information from 125 nm to 240 nm. This has also been recently utilised for residual solvents analysis in APIs and excipients and water determination.[272] The qualitative VUV spectral information can be useful for peak purity assessment (e.g. detect

co-eluting peaks) and peak tracking during early method development (see Figure 14), and confirming peak identity in a sample *vs.* a reference standard and also quantification via spectral deconvolution of co-eluting solvents. However, compared to existing well established GC detectors (e.g. FID, TCD, MS), both VUV and FTIR offer lower sensitivity and are therefore likely to be complementary niche detectors and beneficial for specialised and challenging pharmaceutical applications.

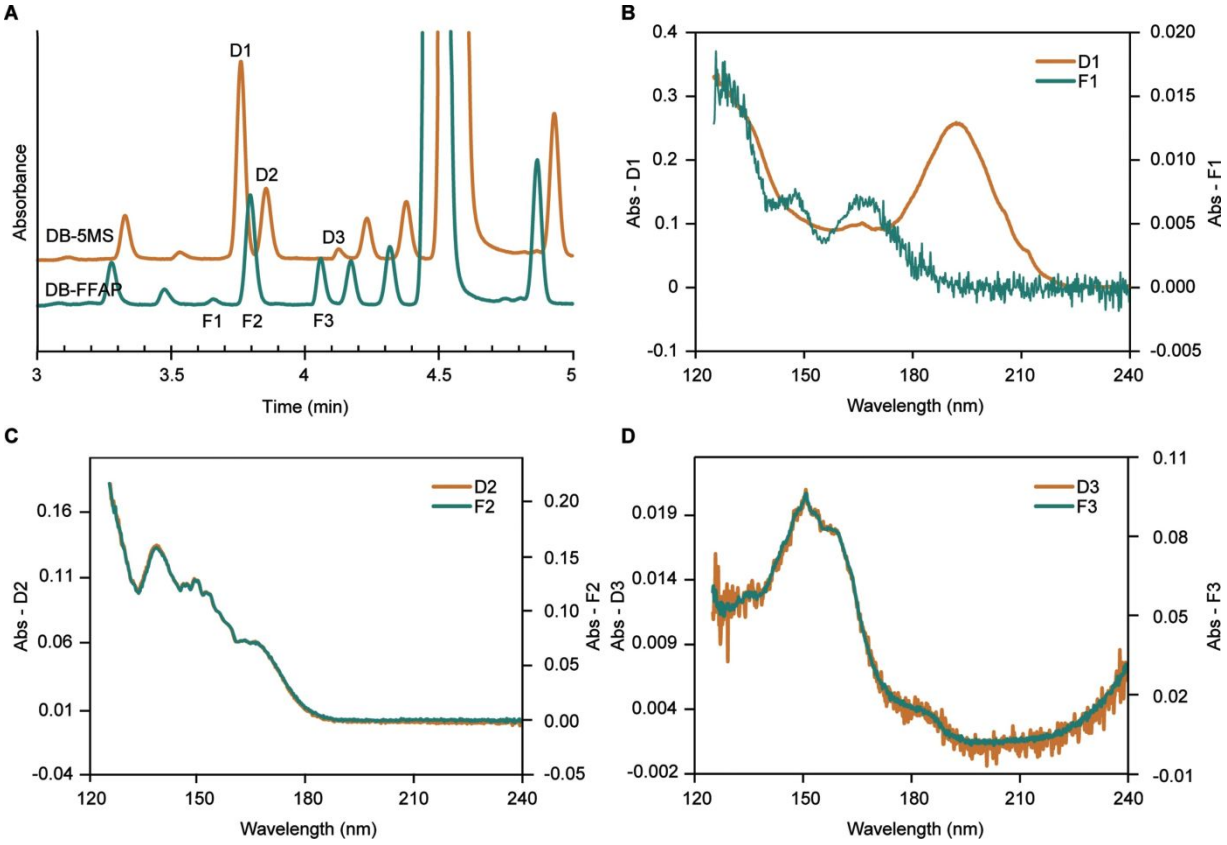

Figure 14. Peak tracking using VUV spectra. A. Overlaid chromatograms of 4-ethoxy-1,1,1-trifluoro-3-buten-2-one acquired on a DB-5MS column and a DB-FFAP column. B. VUV spectra for peak D1 and F1; C. VUV spectra for peak D2 and F2; D. VUV spectra for peak D3 and F3. Reprinted from J. Chromatogr. A, Vol. 1511, J. Zheng, C. Huang, S. Wang, Challenging pharmaceutical analyses by gas chromatography with vacuum ultraviolet detection, pp 185-190 (ref. [273]). Copyright 2018, with permission from Elsevier.

Recent innovations in GC–MS technology has also led to the introduction of a number of high resolution GC–MS systems for structural elucidation and trace analysis.[274] The capability of GC coupled to HRMS for structural characterization to deliver process development and understanding has been demonstrated by several groups.[275,276] Baldwin and co-workers used accurate mass GC–electron ionization (EI)-MS and GC–chemical ionization (CI)-MS data to accurately characterize and quantify low level impurities in starting materials.[276] Characterization of materials and impurity identification and tracking are essential parts of process and product understanding, and quality control for patient safety and adherence to international guidelines.

Also GC–MS and more recently GC-HRMS play vital roles in the characterization and quantitation of extractables and leachables (E&L) that may result from pharmaceutical packaging materials and devices. GC-HRMS has proved to be a key technique in E&L profiling using targeted (*vs.* references/libraries) and non-targeted screening (fully scan). High chromatographic resolution, fast data acquisition, high mass accuracy combined with powerful software and databases/libraries help identify and quantify impurities with higher confidence. Recent work by Zdravkovic *et al.* also used GC-MS and determined relative response factors (RRFs) and retention index values for over 150 diverse organic compounds and generated a database, which they proposed is suitable for use in intra-laboratory extractable screening studies.[277]

GC and MS play an important roles in the quantitation of genotoxic impurities (GTIs) that may result from devices used within the pharmaceutical industry (see section 11).[228–232] Many GTIs are volatile and have a no, or limited UV chromophore (e.g. Alkyl halides) or they can be derivatized to lower the required elution temperature. Therefore, owing to the high resolving power, extra sensitivity and simplicity, GC with a range of detectors (FID, ECD, MS) plays an equal or more important role to LC & LC-MS. Advances in automated and multipurpose sample preparation equipment (e.g. multifunctional auto-samplers, liquid sample preparation, liquid-liquid extractions, Solid Phase Micro Extraction (SPME), Stir Bar Sorptive Extraction (SBPE) etc.) have also facilitated the wider use of GC as part of high performance and high productivity workflows in pharmaceutical analysis.

15. Conclusion and future perspectives

There is no doubt that chromatography will remain the gold standard analytical strategy in the pharmaceutical industry. Indeed, there have been many recent revolutions in terms of the instruments, stationary phases, and chromatographic modes. This review highlights some of the challenges commonly encountered in the pharmaceutical industry and the available solutions. Among the most common problems that analysts face, we can list the determination of genotoxic impurities at very low concentration levels, the strong analytical requirements of cleaning validation procedures, the implementation of PAT tools to efficiently design, control and analyze drugs, the necessity to analyze increasingly complex samples, such as API with multiple chiral centers or new protein biopharmaceuticals formats, and the need for greener chromatographic methods. To deal with all of these difficult analytical questions, numerous chromatographic solutions are generally implemented, including the use of method development software, fast-LC, 2D-LC, SFC, HILIC, LC-MS, GC and GC-MS.

Besides these above-mentioned mature solutions, there have also been many recent additional innovations, which are not discussed in this review, but they present some interesting perspectives for the future. Among them, we can cite miniaturized LC, including the development of slip flow chromatography, pillar array columns, microfluidic chip-based LC, and more reliable nano-LC systems from the major providers of instrumentation. Even if such LC formats are not yet widespread in the pharmaceutical industry, they have begun to attract attention and could probably represent the future of LC. In addition, it is also expected that MS will expand even more as a detection method for pharmaceutical applications (even in QC laboratories) thanks to the commercialization of easy-to-use and low-cost single quadrupole devices. Finally, there should probably be an increasing number of protein biopharmaceuticals to analyze in the coming years, as these biological drugs are now widely used for the treatment of numerous diseases and even for the prevention of episodic and chronic migraines.[278] For this type of demanding application, 2D-LC in combination with MS and even IM-MS[279] will certainly continue its expansion.[24,136,280]

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