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Recent advances in chromatography for pharmaceutical analysis

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KEYWORDS: multidimensional chromatography, fast chromatography, supercritical fluid chromatography, process analytical technology, hydrophilic interaction chromatography, protein biopharmaceuticals, genotoxic impurities, cleaning validation, green chromatography, recent advances in gas chromatography.

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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

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3 attractive for environmental, food, and chemical analyses, where productivity needs to be
4 increased due to the large number of samples. However, the main driving force for
5 developing fast separations is the pharmaceutical industry. Indeed, enhanced productivity
6 and reduced costs are particularly needed during the drug discovery and development
7 processes in applications such as quality control, pharmacokinetics and drug metabolism.
8 Typical pharmaceutical separations can be performed in 2 – 10 min intervals with current
9 UHPLC technology, and complete method development can be carried out in 1 – 2 days.
10 However, it is important to keep in mind that detectors, connection tubing, gradient mixers
11 and mobile phase preheaters had to be redesigned to be compatible with the very narrow
12 peaks obtained by these very efficient small columns. When working with UHPLC columns of
13 50 × 2.1 mm packed with sub-2 μm particles instead of regular HPLC columns of 150 × 4.6
14 mm packed with 5 μm particles, the analysis time can be decreased by a factor of 5 – 9,
15 while maintaining an equivalent kinetic performance.⁵

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

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

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

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24 Another possibility to improve separation times is to work with very short columns (≤ 1 cm)
25 packed with very efficient particles and run steep gradients. However, the handling of poorly
26 retained and very narrow peaks in the domain of conventional HPLC/UHPLC is currently
27 hindered by extra-column dispersion and data sampling rates. Obtaining ideal
28 chromatographic outputs from very efficient columns and fast eluting peaks is hindered by
29 several factors. The shape of the injector pulse, the flow distribution pattern of the inlet and
30 outlet frits, diffusion and mixing in plumbing unions, flow profiles in the connector tubing and
31 the data sampling rate, all affect the true peak shape and width in deleterious ways.¹⁷
32 Despite these physical limitations, a recent study demonstrated the potential of performing
33 subsecond separations using guard columns, which were 5 mm long standard bore columns
34 packed with 2.7 μm SPPs, on a modified commercial UHPLC system.¹⁸ To operate the
35 UHPLC instrument at the highest flow rate possible (5.0 mL/min), the in-line filter was
36 removed. Several modifications were made to a commercial LC system to decrease the
37 extra-column volume and band broadening. The raw chromatographic signals were further
38 processed by deconvolution and peak fitting and finally transformed with a power function.
39 This setup enabled the separation of 2 – 3 compounds in the second range, operating the 5
40 mm columns at a rate of 5 mL/min on the optimized system. Figure 1 shows an example of a
41 1.2 sec separation of 3 acidic compounds.
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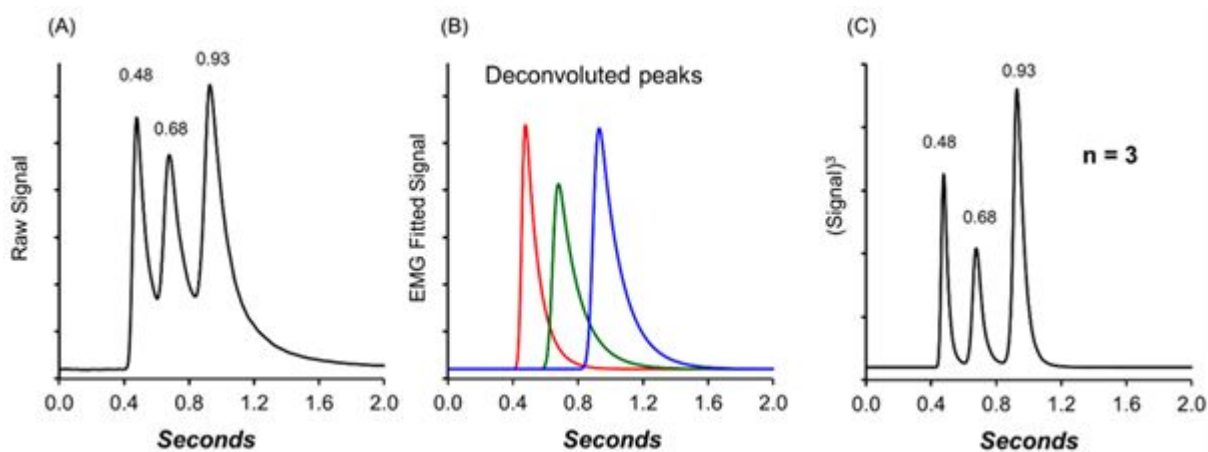


Figure 1. Application of a power transformation to the subsecond chromatography of three components (mellitic acid, 2,3-dihydroxybenzoic acid, and 4-aminosalicylic acid). (A) The original subsecond chromatogram. (B) Deconvolution of the chromatogram into three exponentially modified Gaussian peaks. (C) 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

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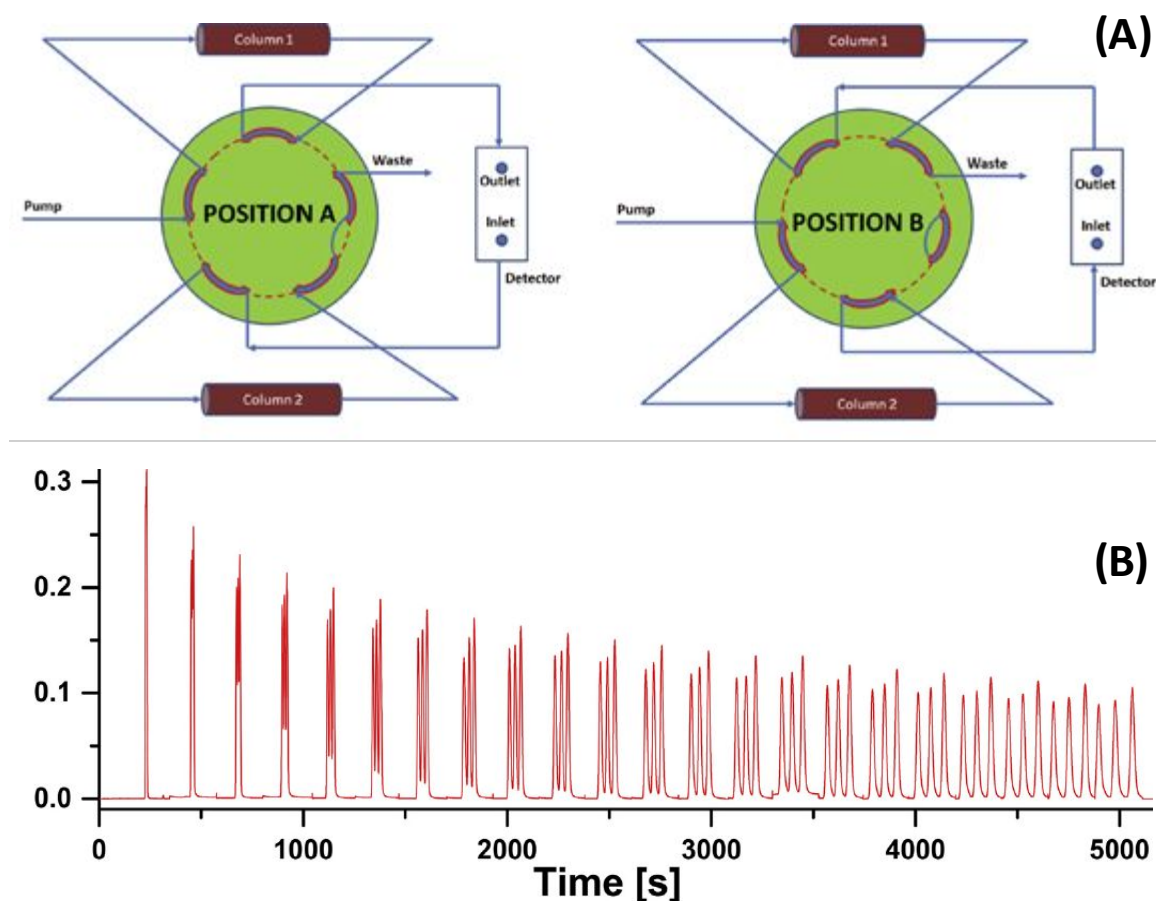


Figure 2. Schematic representation of a twin-column recycling separation system (A) and chromatograms obtained with $n=1$ to $n=22$ recycling cycles for a benzene isotope mixture (B). Reprinted from *J. Chromatogr. A*, Vol. 1524, Gritti, F.; Besner, S.; Cormier, S.; Gilar, M. Applications of high-resolution 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 Carr²⁴ 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.

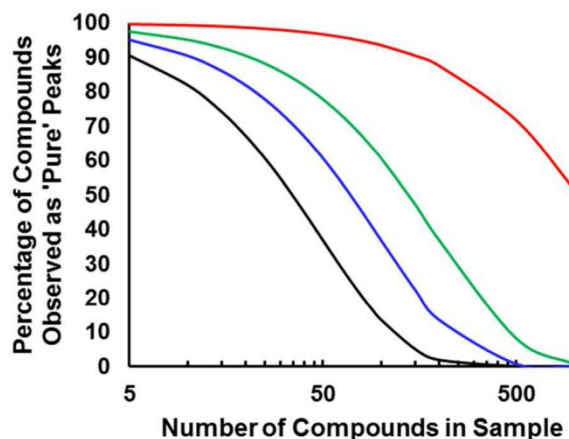


Figure 3. Percentage of compounds that are resolved as a single chromatographic peak (minimum resolution of 1.0) as a function of the number of compounds in the sample and with an effective peak capacity of 100 (black), 200 (blue), 400 (green) and 3000 (red). This behavior is only true when having a 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

	² RP	² NP	² HILIC	² HIC	² IEX	² SEC-Aq	² SEC-Or	² Ag	² Chiral	² Affinity	² SFC
	F ⁺ H ⁺ Q ⁺ M ⁺	F ⁻ Q ⁻	M ⁺ Q ⁻	F ⁻ H ⁻ M ⁻ Q ⁻	M ⁻ Q ⁻ S ⁻	F ⁻ H ⁻ I ⁻	F ⁻ H ⁻ I ⁻	F ⁻ Q ⁻ S ⁻	F ⁻ I ⁻ S ⁻	H ⁻ Q ⁻ S ⁻	F ⁻ H ⁻ M ⁻
¹ RP H ²⁺	E O ⁺ P ⁺ X ⁺	B O ²⁺ X ²⁻	B O ²⁺ X ⁺	B E O ⁻ P ⁻ X ⁻	O ⁺	A E O ⁺ P ⁺ X ⁺	A E O ⁺	B O ²⁺ X ⁻	O ²⁺	O ²⁺ X ⁺	B O ²⁺ X ⁻
¹ NP H ⁺	B O ²⁺ X ²⁻	O ⁻ P ⁻ X ⁺	O ⁻ P ⁻ X ⁻	B O ²⁺ P ⁻ X ²⁻	O ²⁺	O ²⁺ X ²⁻	O ²⁺ P ⁺ X ⁺	O ⁺ X ⁺	O ²⁺	O ⁺ X ²⁻	O ⁻ X ²⁺
¹ HILIC H ⁺	B O ²⁺ P ⁺ X ⁺	B O ⁻ X ⁻	O ⁻ X ⁺	B O ²⁺ P ⁻ X ⁻	O ⁺ X ⁺	O ²⁺ P ⁺	A O ⁺ X ⁺	B O ⁻ X ⁻	O ²⁺	X ⁻	X ⁺
¹ HIC H ⁻	E O ⁻ X ²⁺	B O ²⁺ P ⁻ X ²⁻	B O ²⁺ X ⁻	O ²⁻ P ²⁻	B O ⁺ P ⁻ X ²⁺	O ²⁺ P ⁻ X ²⁺	A O ⁺ P ⁻ X ⁻	B O ²⁺ P ⁻ X ²⁻	O ²⁺ P ²⁻	O ⁺ X ⁺	O ⁺ P ²⁻ X ²⁻
¹ IEX H ⁻	E O ⁻ P ⁺ X ²⁺	B O ²⁺ X ²⁻	B O ⁺ X ⁻	B O ⁺ P ⁻ X ²⁺	B X ⁻	O ⁺ X ²⁺	A O ⁺ P ⁻ X ⁻	B O ⁺ X ⁻	O ²⁺	O ⁺ X ⁺	O ⁺ X ²⁻
¹ SEC-Aq H ²⁺	E O ⁺ P ⁺ X ²⁺	B O ²⁺ X ²⁻	B O ²⁺ X ⁻	B O ⁺ P ⁻	O ⁺ X ²⁺	O ²⁻ P ²⁻	A O ²⁻ P ²⁻ X ²⁻	O ²⁻ X ²⁻	O ²⁺ P ⁻	O ²⁺ X ⁺	E O ²⁺ P ⁻ X ⁻
¹ SEC-Or H ²⁺	B ²⁻ O ⁻ X ⁻	B O ²⁺ X ⁺	O ⁺ X ⁺	B O ⁺ P ⁻ X ²⁻	B O ⁺ P ⁻ X ⁻	O ²⁻ P ²⁻ X ⁻	O ²⁻ P ²⁻	O ²⁺ X ⁺	O ²⁻ P ⁻	O ²⁺ P ²⁻ X ⁻	O ⁺ P ⁻ X ⁺
¹ Ag H ⁻	B O ²⁺	O ⁺ X ⁺	O ⁺ X ⁺	B O ²⁺ P ⁻ X ⁻	O ²⁺ X ⁻	O ²⁺ X ⁻	O ²⁺ X ⁻	O ²⁻ P ²⁻	O ²⁺	O ²⁺ X ²⁻	O ⁺ X ⁺
¹ Chiral I ⁻	O ²⁺	O ²⁺	O ²⁺	O ²⁺ P ²⁻	O ²⁺	O ²⁺ P ⁻	O ²⁺ P ⁻	O ²⁺	O ²⁻ P ²⁻	O ²⁺	O ²⁺
¹ Affinity H ⁻	O ²⁺ P ⁻ X ⁺	B O ²⁺ P ⁻ X ⁻	B O ²⁺ P ⁻	O ²⁺ P ⁻	O ⁺ P ⁻ X ⁺	O ²⁺ P ⁻ X ⁺	A O ²⁺ P ²⁻ X ²⁻	B O ²⁺ P ⁻ X ⁻	O ²⁺ P ⁻	O ⁻ P ²⁻	O ⁺ P ⁻ X ²⁻
¹ SFC H ⁻	E O ²⁺ X ⁺	O ⁻ X ⁺	E O ⁻	O ²⁺ P ³⁻	O ²⁺ X ⁺	O ²⁺ P ²⁻ X ²⁺	O ²⁺ X ²⁺	O ⁺ X ⁺	O ²⁺	O ²⁺ X ⁻	E O ⁻ X ²⁺

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

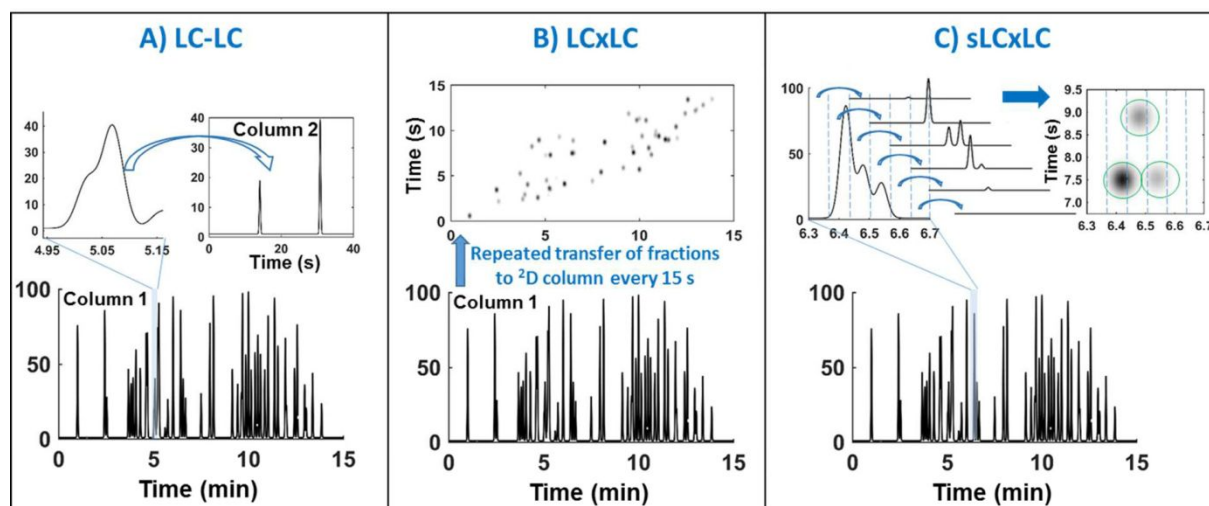


Figure 5. Visual comparison of different implementations of 2D-LC. A) LC-LC (heart-cutting 2D-LC): a single fraction is injected into the 2D column. In multiple heart-cutting (mLC-LC, not shown), more than one region of the 1D effluent would be injected onto the 2D column. B) LC \times LC (comprehensive 2D-LC): the entire 1D effluent is sequentially injected. C) sLC \times 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 (α -1-acid glycoprotein) in the second dimension, was used for the successful separation of ketoprofen enantiomers.³⁵ The experimental setup included factorial

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3 designs as a mathematical approach to allow for the easy optimization of both separation
4 conditions, with a focus on the compatibility of the mobile phases.
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7 Beyond the coupling of orthogonal RP selectivities, heart-cutting 2D-LC was also applied to
8 other LC modes. For example, Wang *et al.*³⁶ proposed the characterization and quantification
9 of polar excipients in protein formulation samples by heart-cutting SEC-HILIC 2D-LC.
10 Namely, histidine degradation was tracked in complex sample matrices, and its main
11 degradant was identified as trans-urocanic acid. In addition, quantification of the degradant
12 was achieved thanks to the combining of the 2D-LC setup with stable-isotope labeling MS. In
13 a different setup, Luo *et al.*³⁷ used RPLC as the second dimension in a heart-cutting 2D-LC
14 MS system to perform an in-line desalting step prior to MS detection. Separation of the
15 compounds of interest (a synthetic peptide containing thirty amino acid residues) was
16 performed exclusively by the first RPLC dimension using an NaClO₄ additive in the mobile
17 phases. NaClO₄, which is an MS-incompatible ion-pairing agent, was then separated in the
18 second dimension and discarded prior to the entry of the effluent of the second dimension
19 into the MS. Several examples were provided to validate the versatility of the approach, and
20 the separation of complex mixtures of peptides containing impurities and positional isomers
21 was extensively discussed.
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31 Compared to the heart-cutting 2D-LC mode, the multiple heart-cutting approach (mLC-LC)
32 allows for more than one region of the first dimension to be injected into the second
33 dimension. An innovative application of mLC-LC for the analysis of pharmaceutical impurities
34 was proposed by Zhang and coworkers, who used a primary column in the first dimension
35 and six orthogonal columns in the second dimension to enhance the flexibility and selectivity.
36 In this configuration, heart cuts from the first dimension were directed into “parking” loops,
37 where they remained until being injected into the second dimension. The tested approaches
38 involved an mRPLC-RPLC setup, including phenyl and C18 as the stationary phases, and
39 were able to reveal hidden degradation products that coeluted with the main drug peak. In
40 addition, an mRPLC-HILIC setup was able to resolve polar component that was poorly
41 retained in the first dimension. Through mLC-LC consisting of SEC and ion-pairing RPLC,
42 Ouyang *et al.*³⁸ were able to identify and assign more than 80 and 120 oligosaccharides,
43 respectively, from the low molecular weight heparins (LMWHs) nadroparin and enoxaparin.
44 In particular, the 2D-LC approach was emphasized as being of the utmost importance for
45 profiling analysis, where LMW components of either the same or different sizes, but having
46 different charges and polarities, are resolved in the same analysis.
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57 In contrast to LC-LC and mLC-LC, the comprehensive 2D-LC approach (LC × LC, Figure 5B)
58 allows the entire effluent of the first dimension to be injected onto the second dimension. This
59 approach is often used for the separation of very complex samples (especially in *-omics*
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3 applications) or, more generally, when the goal is to gain as much information as possible
4 from the samples. Sarrut and coworkers³⁹ highlighted the pros and cons of this approach by
5 comparing the results of one-dimensional (1D-RPLC) *versus* in-line comprehensive two-
6 dimensional LC (RPLC × RPLC) for optimized, sub-hour separations of complex peptide
7 samples. It was shown that, within an analysis time of 60 min, the same peak intensity was
8 observed with both techniques but a 3-fold lower injected volume was used in RPLC ×
9 RPLC. In addition, coupling to an MS was more advantageous when performing RPLC ×
10 RPLC based on a significant increase in the signal-to-noise ratio (strong noise reduction),
11 which suggests that RPLC × RPLC–MS is a promising technique for peptide identification in
12 complex matrices. Stoll and coworkers⁴⁰ evaluated the impact of comprehensive LC × LC on
13 UV detection sensitivity during the analysis of degraded APIs. A systematic evaluation of the
14 impact of the volume and solvent composition of fractions of the first dimension on the ability
15 of the second dimension to resolve and detect low-abundance compounds was performed.
16 Interestingly, it was found that dilution of the first dimension effluent with a weak solvent prior
17 to injection into the second dimension had the beneficial effect of concentrating the analyte
18 band in the second column, causing an overall improvement in the sensitivity. Corgier *et al.*⁴¹
19 used LC × LC for another interesting application by a proof-of-concept investigation on the
20 potential of the technique for micropreparative separations of simple samples. In this work,
21 the authors⁴¹ investigated the usefulness of the approach for recovering minor components
22 from a simple mixture that can be separated by 1D-LC. A test sample of four compounds
23 was separated by two 1D-LC methods and one LC × LC method. A comparison of the
24 preparative performance of the three methods showed that in-line LC × LC was able to
25 achieve a 12-fold increase in the amount of compounds recovered per injection compared to
26 1D-LC. At the same time, the production rate was increased by a factor of 9.5.

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41 Finally, the selective comprehensive 2D-LC approach (sLC × LC, Figure 5C) allows
42 segments of the first dimension to be comprehensively injected into the second dimension,
43 with the advantage of having the peak volume of the first dimension divided into smaller
44 volume fractions that have less of an impact on the second dimension separation. As
45 reported by Stoll and coworkers,³⁰ sLC × LC is generally applied to the determination of peak
46 purity during method validation as an effective alternative to UV-vis, diode-array, or MS
47 detection. As an example of this application, Venkatramani *et al.*⁴² demonstrated the ability of
48 sLC × LC to resolve structurally similar impurities that eluted in proximity to the main
49 component, with coeluting impurities resolved and detected at levels <0.05% in the presence
50 of the main component. Similarly, Shackman *et al.*⁴³ highlighted the suitability of the sLC ×
51 LC for the determination of peak purity of a pharmaceutical formulation containing multiple
52 active components that possessed diverse physicochemical properties. The analytical
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3 platform allowed for drug product impurity and degradant profiling in the first dimension (a
4 total of 14 targeted fractions were sampled across the first dimension main peak), and the
5 assay of the fractions through a drug substance profiling method in the second dimension.
6 This degree of sampling allowed for the profiling of a coeluting degradant present at a level
7 of 0.2% w/w throughout the main peak.
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11 It is clear that 2D-LC is attracting significant increasing interest and that the inclusion of this
12 technique in the analytical toolbox of pharmaceutical analysis can go beyond increasing the
13 peak capacity, as recently discussed by Haidar Ahmad *et al.*⁴⁴ by highlighting three different
14 applications of 2D-LC in the pharmaceutical industry. First, 2D-LC was applied to match the
15 retention times for peaks of interest under different mobile phase conditions by “transforming”
16 an incompatible MS buffer into a MS-compatible buffer. Collection of the peaks of interest
17 was performed in the first dimension, via a heart-cutting method, and injected into the second
18 dimension, where a volatile LC-MS-compatible buffer was used. The second application
19 involved the development of a 2D-LC method able to simultaneously detect and quantify
20 degradation products in a pharmaceutical material that contained both small and large
21 molecules. Finally, the third application had the goal of using 2D-LC to support stability-
22 indicating methods. Specifically, the purity of separated peaks was assessed using an
23 orthogonal column in the second dimension, which was selected based on the hydrophobic
24 subtraction model.⁴⁴
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34 Due to the obvious benefits that 2D-LC offers in several applications of pharmaceutical
35 analysis, it is reasonable to think that its implementation in pharmaceutical laboratories will
36 be wider and of the utmost interest in the near future. However, it is also clear that LC-MS
37 (involving benchtop MS devices as the ones reported in Section 7) should not be neglected
38 in such applications, as it can be considered a 2D analytical procedure. Finally, it is worth
39 mentioning that several 2D-LC approaches have also been applied in biopharmaceutical
40 analysis due to the structural complexity of the samples. However, for the sake of clarity,
41 relevant examples related to this field will be discussed in Section 6.
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49 **4. Supercritical fluid chromatography of drugs**

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51 Supercritical fluid chromatography (SFC) is an analytical technology originally developed in
52 the 1960s. When pressurizing and heating some fluids beyond their critical point
53 (supercritical state), they exhibit unique behavior as chromatographic eluents.⁴⁵ Indeed, the
54 viscosity and diffusivity of such fluids are very close to those of a gas, resulting in high kinetic
55 performance at modest pressures, while the density and solvating power are equivalent to
56 that of a liquid, providing good solubility of the analytes. Despite these benefits, the
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3 pharmaceutical industry showed limited interest in SFC early on and continued to use well-
4 established GC and LC techniques. Thus, SFC is often perceived as a niche technique and
5 remains a reference technique for chiral separations and preparative scale applications⁴⁶ in
6 the pharmaceutical industry. However, in the last five years, ⁴⁷ the technique has seen real
7 change and a renewed interest for use in analytical achiral applications in the pharmaceutical
8 analysis community. This was mainly driven by (i) significant modifications of the mobile-
9 phase components and compositions, (ii) introduction of innovative stationary-phase
10 chemistries and dimensions, and (iii) commercialization of state-of-the-art systems with a
11 design that is based on recent UHPLC instruments, thus offering drastic improvements in
12 instrument reliability and performance.⁴⁸ In this section, the applicability of SFC in
13 pharmaceutical analysis, including the determination of drugs in formulations and biofluids, is
14 critically discussed.

15
16 One of the most significant changes in SFC, from its early inception to modern times, has
17 taken place in the mobile-phase components and compositions.⁴⁸ In particular, the inclusion
18 of organic solvents and the use of additives (acids, bases or salts) has dramatically extended
19 the range of applicability of SFC to include more polar and ionizable compounds. In this
20 context, Galea *et al.* investigated the possibility of blending several organic solvents (i.e.,
21 ethanol, methanol, isopropanol, THF, acetonitrile) rather than using only methanol (most
22 common solvent) to further improve the selectivity of SFC during the method development of
23 drugs. However, the nature of the modifier leads to only minor changes in terms of the
24 elution order, retention and selectivity in comparison with changes to the stationary phase⁴⁹
25 of achiral SFC. West *et al.* have recently investigated the effects of additives on SFC drug
26 analysis. They demonstrated that additives in the usual concentration range (0.1% or 10 – 20
27 mM) do not modify the polarity in the immediate vicinity of the probe.⁵⁰ They also used color
28 indicators to determine an apparent pH of 5 for carbon dioxide–methanol mobile phases.
29 They finally showed that adding a basic additive had little influence on the apparent pH, while
30 acidic additives can further decrease the pH below 1.7.

31
32 In recent years, several groups have published works related to finding the ideal sample
33 diluents for modern SFC. If the sample diluent is adequately selected, it becomes possible to
34 inject larger volumes in SFC, leading to a substantial improvement in sensitivity,⁵¹ which is
35 particularly relevant for the impurity profiling of drugs. As illustrated in Figure 6, it was
36 demonstrated that aprotic solvents, such as methyl tert-butyl ether, dichloromethane,
37 acetonitrile or cyclopentyl methyl ether, are well suited for the injection of large volumes in
38 modern SFC, while MeOH was generally the worst option. However, the stationary-phase
39 chemistry may also have a strong impact, and some of these diluents did not perform equally
40 well on each column. This was probably related to competitive adsorption of the analyte and
41 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.

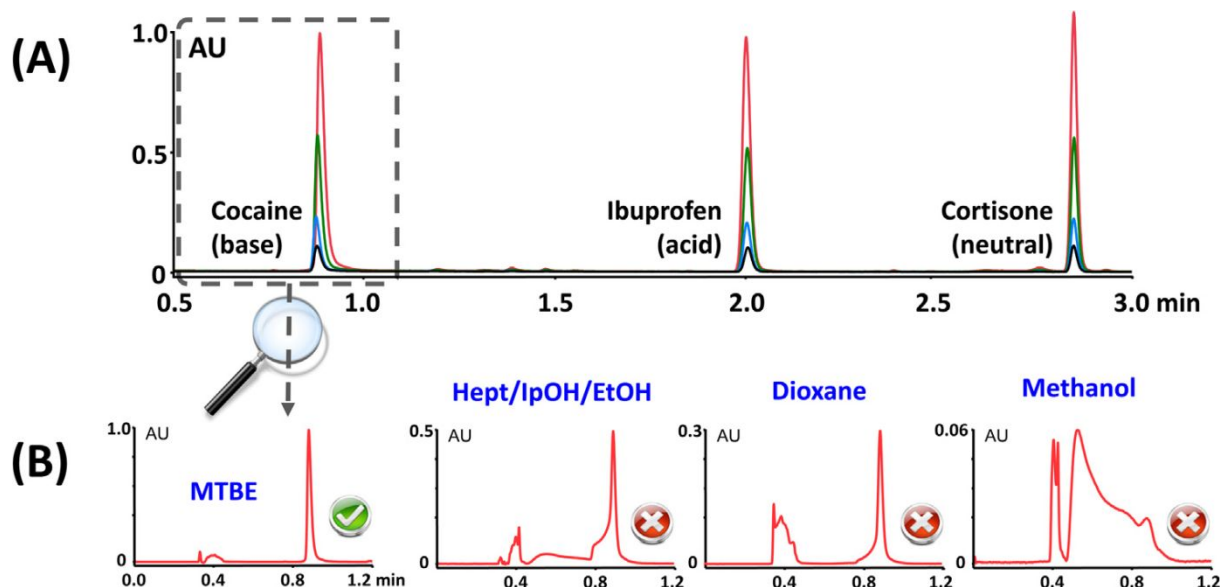


Figure 6. (A) Chromatograms obtained from the injection of 1 (black), 2 (blue), 5 (green) and 10 (red) μL of a mixture of cocaine, ibuprofen and cortisone at 100 $\mu\text{g}/\text{mL}$ each in MTBE on 2-PIC. (B) Injection of 10 μL of a cocaine solution at 100 $\mu\text{g}/\text{mL}$ with four different 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. Guillaume, 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, UPC² 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

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

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

20
21 Another important trend in SFC is its coupling to mass spectrometry (MS), since this detector
22 has become the gold standard for the determination of trace compounds in complex matrices
23 due to its high specificity, sensitivity and quasi-universality.⁵⁹ As shown in Figure 7, various
24 robust and flexible SFC-MS interfaces have been proposed to (i) manage the compressibility
25 of the SFC mobile phase when it is no longer under the control of the backpressure, (ii)
26 improve the ionization yield in the ESI mode when the mobile phase has a large percentage
27 of CO₂, and (iii) maintain the chromatographic integrity in terms of retention, selectivity and
28 efficiency.⁶⁰

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

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

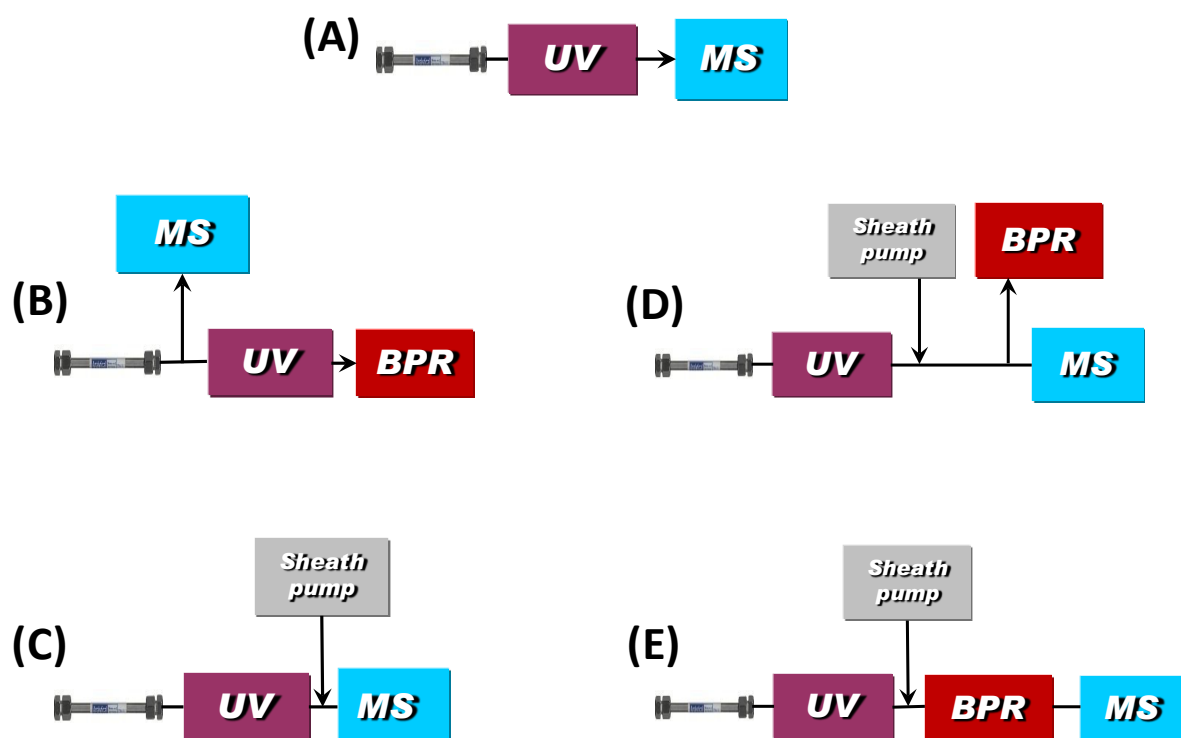


Figure 7. Schematic representations of the five most common SFC–MS interfaces. (A) “direct coupling” interface, (B) “pre-UV and BPR splitter without sheath pump” interface, (C) “pressure control fluid” interface, (D) “pre-BPR splitter with sheath pump” interface, and (E) “BPR and sheath pump with no 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 (ref⁶¹). 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 CO₂. In the last 10 – 15 years, the application of SFC has been

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3 extended to include the analysis of moderately polar substances, such as drugs, by using up
4 to 30 – 40% MeOH in the mobile phase. More recently, SFC has also been applied to the
5 analysis of highly polar substances, thanks to the use of very high organic solvent
6 proportions (up to 100%) and significant amounts of volatile salts in the mobile phase. In
7 these cases, supercritical conditions are no longer observed, but this suggests that SFC has
8 the ability to analyze mixtures with an extended polarity range, which includes nonpolar,
9 polar and very polar compounds, in a single run when applying a gradient from pure CO₂ to
10 pure methanol. In these particular conditions, the term SFC is not technically correct, since
11 the mobile phase is no longer under a supercritical state. This approach has been addressed
12 as unified chromatography (UC) or enhanced-fluidity liquid chromatography (EFLC) and is
13 increasingly becoming widely used. Various applications have been reported for the
14 simultaneous analysis of liposoluble and hydrosoluble vitamins,⁶⁹ the determination of
15 numerous environmental pollutants having an extended range of polarity,⁷⁰ the simultaneous
16 determination of lipophilic and hydrophilic metabolites (metabolomics),⁷¹ the analysis of
17 numerous pesticides⁷² covering a wide polarity range, and, more surprisingly, the analysis of
18 proteins (the achieved performance were, however, far from those reported in Section 6
19 using LC conditions).⁷³

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

41 **5. HILIC for polar and ionizable compounds**

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

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

22
23 The mobile-phase additive plays a key role in HILIC, since it allows for suitable peak shapes
24 and low retention-time variability by stabilizing the charge of the ionizable solutes and
25 ionogenic groups on the surface of the stationary phase.⁹⁰ Even when conventional acids
26 (i.e., formic acid and acetic acid) have been used in HILIC, they do not provide sufficient
27 ionic strength in high concentrations of ACN to produce good peak shapes.⁹¹ Therefore, the
28 majority of modern HILIC separations are conducted with ammonium formate and
29 ammonium acetate buffers, with a mobile phase pH ranging between 3 and 6. Interestingly,
30 Heaton *et al.* demonstrated that the use of a citrate buffer, rather than an ammonium formate
31 buffer, at pH 3 improved the peak shapes of catecholamines due to a reduction in the
32 negative effects of metals in the system by their preferential complexation.⁹² More recently,
33 retention studies were also performed at a pH below 3 using either TFA or stronger ion-
34 pairing reagents, such as heptafluorobutyric acid and methanesulfonic acid. Some
35 unexpected behavior was noticed, and in particular, TFA use resulted in anion exchange
36 properties, which contrasts with the cation exchange typically found when using an
37 ammonium salt buffer.⁹³ Therefore, when using 0.1% TFA as the mobile phase additive, the
38 elution order of drugs was very different from that observed with ammonium formate at pH 3.

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40 The nature of the sample diluent is another important parameter that needs to be adequately
41 controlled to achieve sharp peaks in HILIC. This was demonstrated some years ago by Ruta
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3 *et al.*⁹⁴ and continues to attract attention today.⁹² To limit deterioration of the column
4 efficiency, the sample should ideally be diluted in a high proportion of aprotic solvent (at least
5 80% ACN). Small increases in the water content of the injection solvent may be acceptable,
6 if the injection volume remains low (below 1% of the column volume), to accommodate
7 compounds that are poorly soluble in an acetonitrile-rich mobile phase. Obviously, the
8 detrimental effects of increased injection volume and solvent mismatch were more serious
9 for solutes with smaller retention factors.⁹² Recently, Gritti *et al.* highlighted the importance of
10 the sample diluent in the analysis of cetirizine (a second-generation antihistamine drug)
11 when using a recommended USP HILIC method.⁹⁵ Indeed, a significant peak deformation
12 was noticed due to the higher concentration of a strong solvent (water) in the sample diluent
13 compared to the mobile phase. The concentration profiles of cetirizine and water were
14 calculated using the equilibrium dispersive model of chromatography. Based on this work, it
15 appears that various modifications should be brought to HILIC methods to maintain peak-
16 shape integrity. First, the water concentration of the diluent should not exceed that of the
17 mobile phase. If that is not possible, the retention factor of the analyte should be at least 2-
18 fold smaller than that of the water perturbation. If this is not possible, the amount of pre-
19 column sample dispersion should be increased until the decrease in efficiency is too great.
20 Finally, if none of the above mentioned technical solutions works, the injection volume can be
21 decreased, as long as the impurities remain detectable.

22
23 To further extend the applicability of HILIC to a wider range of pharmaceutical compounds,
24 various new HILIC columns have appeared in research papers every year.^{87,96} Silica-based
25 materials remain, by far, the most widely used stationary phases for HILIC. In particular, bare
26 and amide-bonded silica, zwitterionic and diol groups are the most common phases used to
27 perform HILIC separations of pharmaceutically relevant compounds. HILIC phases can be
28 intuitively categorized based on their possible ionic interactions, including neutral (e.g.,
29 amide, cyano, diol), positively charged (e.g., amino, imidazole, triazole), negatively charged
30 (e.g., polyaspartic acid, bare silica) and zwitterionic (e.g., sulfobetaine or peptide) phases.⁸⁷
31 In addition to the ionic selectivity, the hydrophilic selectivity also plays an important role and
32 depends on the thickness of the water layer. For instance, the amide and zwitterionic phases
33 possess large water layer thicknesses, while bare silica possesses the smallest.⁵⁹ In addition
34 to these well-known stationary phases, some additional HILIC stationary phases were
35 recently described. A novel polyacrylamide-based silica stationary phase was prepared by
36 Cai *et al.*,⁹⁷ using a two-step synthesis method, for the analysis of carbohydrates using
37 alcohols as the mobile phase rather than acetonitrile (thanks to the high hydrophilicity of this
38 phase), which was beneficial for green analysis and the purification of polar compounds. A
39 multifunctional phase, synthesized from L-isoleucine and 4-phenylbutylamine, was used as a
40 mixed-mode HILIC/RPLC phase.⁹⁸ This new stationary phase was found to be particularly
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3 well suited to the analysis of nucleotides/nucleosides and for the simultaneous analysis of
4 weakly polar and nonpolar solutes. Hou *et al.*⁹⁹ prepared a porous graphitic carbon (PGC)
5 stationary phase with quaternary ammonium-polyvinyl alcohol-mixed functional groups for
6 HILIC operation. This phase offered typical HILIC characteristics but exhibits very different
7 selectivity compared to other HILIC phases and bare PGC. In addition, this phase was
8 superior to silica-based HILIC phases in terms of pH tolerance (2.1 – 12.7) and bleeding.
9 Qian *et al.*¹⁰⁰ also suggested a valuable solution to reduce the column bleeding that results
10 from silica dissolution. For this purpose, multiple layers of a polyvinyl alcohol cross-linked
11 with glutaraldehyde were coated onto silica gel, leading to a 20-fold reduction in bleeding
12 compared to bare silica.
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19 When combining HILIC and MS, a substantial average increase in the sensitivity by a factor
20 of 7 to 10 can be expected compared to RPLC-MS.¹⁰¹ This sensitivity increase of HILIC was
21 obviously explained by the difference in acetonitrile concentration during the elution of drugs
22 (on average, 30% ACN in RPLC and 80% ACN in HILIC), leading to better analyte
23 desolvation. However, this high proportion of organic solvent also favorably influenced
24 ionization by modifying the mobile-phase pH and solute pK_a . As such, the ionic character of
25 the analytes in solution also plays an important role in explaining the sensitivity enhancement
26 of HILIC. However, the sensitivity improvement of HILIC is strongly dependent on the design
27 of the ESI source.⁸⁷ In a recent systematic study, a large majority of pharmaceutical
28 compounds were detected better with HILIC vs. RPLC when using a Waters MS. On a
29 modern Agilent triple quadrupole instrument, the performance drastically depended on the
30 mobile-phase pH and flow rate conditions, and the benefits of HILIC were only noticed at low
31 flow rates and intermediate pH (pH = 6). Surprisingly, on the Sciex instrument, most of the
32 drugs were detected better with RPLC vs. HILIC. As a general comment, the increase in
33 sensitivity observed in HILIC was less significant with recent LC-MS platforms than with old-
34 generation instruments. Indeed, the improved ESI sources used in newer mass analyzers
35 allow for enhanced evaporation efficiency, and this feature is particularly beneficial for RPLC
36 conditions. In addition to sensitivity, matrix effects in RPLC and HILIC were also
37 systematically studied using a wide range of model pharmaceutical compounds in plasma
38 and urine matrices. When using a nonselective extraction procedure (dilute and shoot or
39 protein precipitation), matrix effects were lower in RPLC vs. HILIC regardless of the matrix. In
40 contrast, HILIC appears to be a valuable alternative to RPLC for plasma and urine samples
41 treated using a selective extraction procedure.¹⁰² More importantly, the compounds
42 influenced by the matrix effects were different in HILIC and RPLC, suggesting that changing
43 the chromatographic method can be an interesting strategy to reduce/eliminate matrix effects
44 in bioanalysis.
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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 (cisplatin¹⁰³ and 5-FU¹⁰⁴). It is also a reference method for the analysis of carbohydrates,^{105,106} amino acids,¹⁰⁷ small peptides¹⁰⁸ 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 metabolites¹¹⁴ (also including some lipids¹¹⁵) 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.

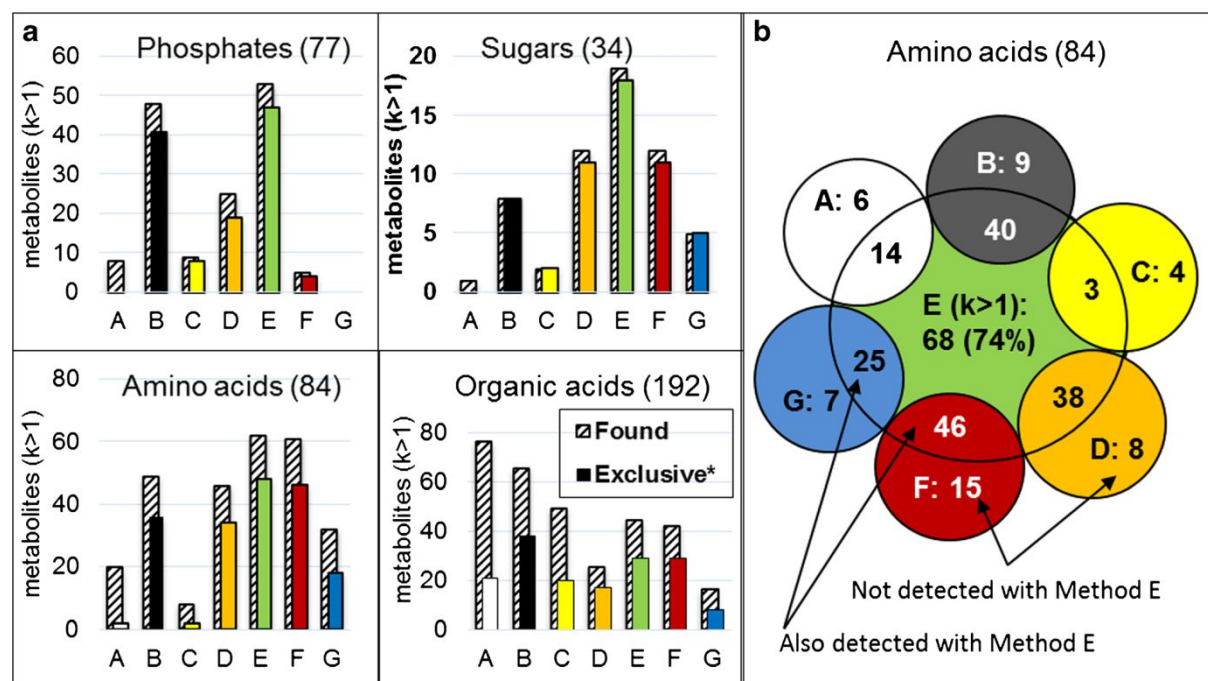


Figure 8. Method A: C18 RPLC column, method C: mixed mode HILIC/RPLC column, methods B and E: zwitterionic HILIC column, Method D and F: neutral HILIC column, method G: amino HILIC column. (a) Coverage of metabolite groups. Shaded bars: retention factor >1, good peak shape. Colored bars: number of metabolites 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

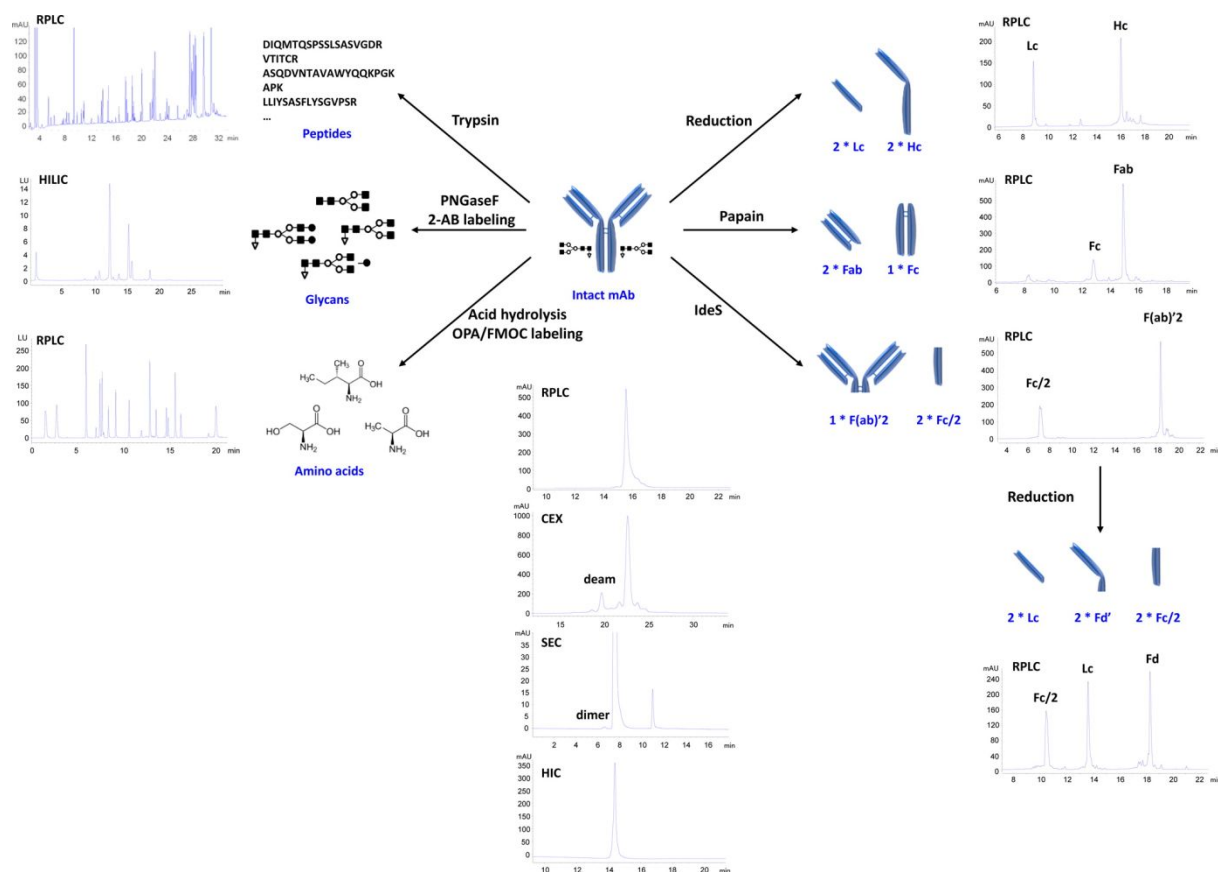


Figure 9. Flowchart of the different LC approaches (RPLC, HIC, SEC, CEX, and HILIC) that are generally used for mAb characterization (trastuzumab as an example) at the protein, peptide, glycan, and amino acid levels. Reproduced from Fekete, S.; Guillarme, D.; Sandra, P.; Sandra, K. *Chromatographic, 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,

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

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

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28 As anticipated in Section 3, 2D-LC has been widely applied to the characterization of protein
29 biopharmaceuticals, especially for mAb and ADC structural characterization at the peptide,
30 subunit and intact levels. The application of comprehensive 2D-LC for peptide mapping of
31 mAbs in both R&D and routine (QA/QC) environments was discussed by Vanhoenacker and
32 coworkers.¹³³ A tryptic digest of trastuzumab was analyzed by 3 different 2D-LC
33 combinations, including CEX × RPLC, RPLC × RPLC, and HILIC × RPLC, with both UV
34 (DAD) and MS detection. The orthogonal information obtained by the application of the
35 different LC × LC approaches allowed for assessing both the identity and purity of the
36 sample. In addition, comparison studies were performed between two different batches of
37 trastuzumab and a biosimilar agent under development that highlighted some differences,
38 such as the increase of a specific deamidation from 8 to 13% and the clipping of the C-
39 terminal lysine that was almost driven to completion in the originator but only removed in
40 87% of the biosimilar molecules. Based on the precision of the method in terms of the peak
41 volume and retention time, the authors were confident about using the approach in future
42 QA/QC testing. Comparisons of originator and biosimilar mAbs were also performed by
43 Sorensen and coworkers at the middle-up level of analysis.¹³⁴ Specifically, 3

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3 reference/biosimilar pairs of mAbs (i.e., trastuzumab, cetuximab and infliximab) were
4 analyzed by CEX × RPLC–HRMS, after which mAb subunits were obtained by either *IdeS*
5 digestion or *IdeS* digestion followed by reduction with dithiothreitol (DTT). Interestingly,
6 despite the size of the subunits, 23 chemically unique mAb fragments were detected in a
7 single sample. Furthermore, direct comparison between the 2D counterplots allowed for a
8 facile assessment of the degree of similarity between the reference and biosimilar
9 samples.¹³⁴ As an extension of this work, in a very recent report, the authors applied HILIC ×
10 RPLC–HRMS to the separation of mAbs at the subunit level to reveal the extent of
11 glycosylation on the Fc/2 and Fd subunits with analysis times on the order of 2 h. In
12 comparison to previous CEX × RP separations of the same molecules, chromatograms from
13 the HILIC × RPLC–HRMS separations were found to have higher resolution and revealed
14 separation of some of the glycoforms that coeluted in the CEX × RP separations.¹³⁵ A more
15 general overview of the potential benefits of 2D-LC over conventional 1D-LC for
16 characterizing therapeutic mAbs is provided by Stoll *et al.*,¹³⁶ where the possibility to increase
17 the resolving power, obtain complementary information from different column selectivities
18 and directly couple the 2D-LC system to MS are extensively discussed. In addition, the
19 suitability of 2D-LC to the characterization of more complex samples, such as ADCs, is
20 anticipated. In this context, several contributions have arisen in recent years, in response to
21 this analytical demand, using heart-cutting,¹³⁷ multiple heart-cutting,¹³⁸ and comprehensive
22 2D-LC.¹³⁹ HIC × RPLC–HRMS was performed by Sarrut *et al.*¹³⁹ to obtain and profile the
23 drug-to-antibody ratio (DAR) of brentuximab vedotin in the first dimension (HIC) with an in-
24 line desalting step performed in the second dimension (RPLC) prior to the coupling with MS
25 that allowed accurate identification of positional isomers. Based on the same approach,
26 Ehkirch and coworkers¹⁴⁰ developed a 4-dimensional platform, consisting of HIC × SEC–IM–
27 MS, for performing in-line characterization of brentuximab vedotin in native conditions. In this
28 configuration, the second dimension (SEC) was exclusively used as a fast desalting step to
29 maintain the non-denaturing conditions of the separation performed in the first dimension
30 (HIC) prior to the accurate identification performed by the IM–MS.
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48 Recently, the thorough and simultaneous characterization of both small and large molecules
49 (drug payloads decorating mAbs in ADC formats) has also stimulated interest in the
50 applications of 2D-LC.^{136,137,139} As practical examples, Venkatramani *et al.*¹⁴¹ performed
51 qualitative and quantitative analysis by high-resolution sampling (HRS) 2D-LC of a key linker
52 drug intermediate used in ADCs. The approach was applied to three groups for comparison
53 and was able to identify 15 impurities coeluting with the linker drug intermediate in the first
54 dimension that were then separated in the second dimension. Similarly, Li and coworkers¹⁴²
55 applied heart-cutting SEC–RPLC to identify and quantify unconjugated small-molecule drugs
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3 and their related small-molecule impurities in ADC samples without the need to perform
4 additional sample preparation. Sandra *et al.*¹⁴³ demonstrated the versatility of heart-cutting
5 and comprehensive 2D-LC for mAb clone selection during mAb and biosimilar molecule
6 development. In this work, the authors combined Protein-A affinity chromatography (first
7 dimension) with SEC, CEX, or RPLC (second dimension), with the goal of simultaneously
8 assessing mAb concentration and critical structural aspects such as aggregation,
9 fragmentation, charge heterogeneity, molecular weight (MW), amino acid sequence and
10 glycosylation. Data were first acquired at the intact protein level in LC–LC mode and then
11 complemented by information derived from peptide mapping performed in LC × LC mode
12 coupled to MS. The versatility of the approach was demonstrated through the analysis of
13 trastuzumab and tocilizumab-producing CHO clones and has the potential to be extended to
14 process optimization.
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23 Beyond applications of 2D-LC MS, another recent advance in LC analysis of
24 biopharmaceuticals has occurred thanks to the introduction to the market of new SEC,
25 RPLC, and HILIC column technologies.
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28 As one example, in recent years, we have been witnessed the translation of SEC methods
29 into ultrahigh performance SEC (UHP-SEC). This has been possible through a focus on
30 column modifications in terms of the particle size, inertness, and column dimensions,
31 resulting in small inert SEC columns packed with sub-3 μm particles. UHP-SEC columns
32 meeting these specifications are currently available from various providers, namely, the
33 AdvanceBio SEC column (2.7 μm, 300 Å) from Agilent, the TSKgel UP-SW3000 column (2.0
34 μm, 250 Å) from Tosoh, the Yarra SEC-X150 (1.8 μm, 150 Å) and the SEC-X300 columns
35 (1.8 μm, 300 Å) from Phenomenex, the Unix (1.8 μm, 200 Å) and the Unix-C columns (1.8
36 μm, 300 Å) from Sepax, and the ACQUITY UPLC Protein BEH SEC column (1.7 μm, 200 Å)
37 from Waters. The practical possibilities and limitations of a few of the above mentioned
38 columns in the field of biopharmaceutical analysis were recently evaluated by Goyon and
39 coworkers,¹⁴⁴ by analyzing several commercial mAbs and ADCs. It was found that the UHP-
40 SEC columns were equivalent in terms of mAb characterization performance, since the
41 average resolution for each column was between 1.9 – 2.1 for the monomer and dimer forms
42 of 10 different commercial mAbs. Furthermore, a drastic increase in the throughput of
43 aggregate analysis was achieved, with analysis times ranging between 3 and 8 min. Despite
44 these kinetic advantages, it appears that uncontrolled secondary interactions still exist with
45 these phases and affect both the elution times and the aggregate recovery. Among these
46 secondary interactions, hydrophobic interactions were generally more prominent than
47 electrostatic interactions, even though fine-tuning of the mobile-phase composition helped to
48 mitigate these effects while maintaining non-denaturing conditions. However, it should be
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3 noted that secondary interactions in UHP-SEC columns are much less pronounced
4 compared to old-fashioned SEC columns packed with 5-10 μm particles. In this context,
5 UHP-SEC columns demonstrated the ability to properly resolve the HMWS of highly
6 hydrophobic structures, such as ADCs, without the need to add organic solvents to the
7 mobile phase.¹⁴⁴ As a side note, having more inert SEC stationary phases may open the
8 doors to a completely new set of applications, including the possibility of direct SEC-MS
9 coupling. This possibility was indeed investigated by comparing UHP-SEC separations
10 obtained with standard (nonvolatile) SEC mobile phases, consisting of 50 mM potassium
11 phosphate and 250 mM potassium chloride, and MS-compatible (volatile) mobile phases,
12 consisting of 100 mM ammonium acetate.¹⁴⁵ Thirty therapeutic proteins, including mAbs,
13 ADCs, Fc-fusion proteins and a bispecific antibody (bsAb), were investigated. However,
14 despite great expectations, it was shown that only the acidic therapeutic proteins ($\text{pI} < 7$)
15 showed comparable separation behavior between the nonvolatile and volatile mobile phases,
16 presumably as a result of the secondary interactions (hydrophobic and electrostatic) still
17 playing an active role in the separations. Nonetheless, the ability of UHP-SEC columns to
18 discreetly function with MS-compatible mobile phases was encouragingly applied as a
19 “helping tool” when performing MS analysis in non-denaturing conditions.¹⁴⁶ Indeed, in the
20 field of native MS, a major drawback is the manual sample preparation to desalt the product
21 prior to MS analysis. Ehkirch and coworkers¹⁴⁶ provided a solution to this issue by suggesting
22 the use of UHP-SEC as an in-line automated buffer exchanger. Interestingly, native MS
23 performed with in-line desalting through UHP-SEC resulted in high-resolution mass spectra
24 and improved mass accuracies compared to manual buffer exchange procedures.

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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 \AA , and 0.25
 μm , respectively) showed optimal resolution for mAb separation¹⁴⁷ 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 \AA , 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 coworkers¹⁰ 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 triggered 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.

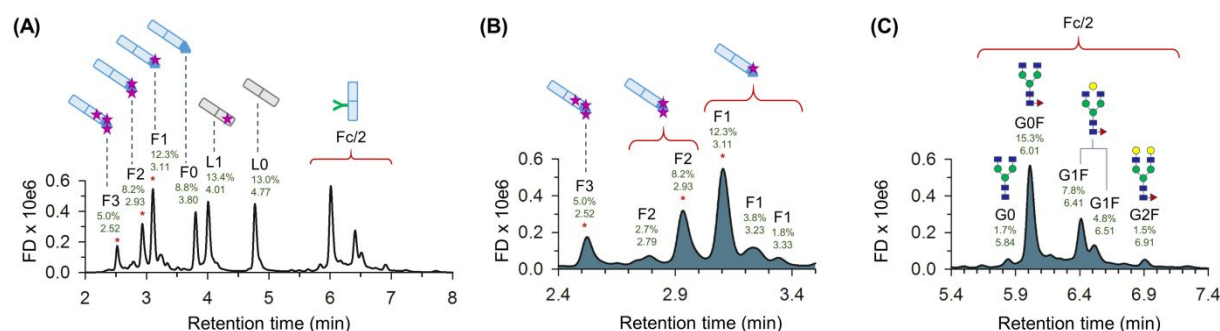


Figure 10. HILIC-MS middle-up analysis of brentuximab vedotin (A) and zoomed in view on the retention windows showing the elution of the drug-loaded Fd' subunits (B) and the Fc/2 glycosylated fragments (C). Peak assignment is accompanied by the relative percentage peak area and retention time. Reprinted from J. Chromatogr. B, Vol. 1080, D'Atri, V.; Fekete, S.; Stoll, D.; Lauber, M.; Beck, A.; Guillaume, 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-workers¹⁵⁰ through the analysis of relevant samples including insulin, interferon α -2b and a mAb, namely trastuzumab. Results showed high orthogonality between HILIC

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

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33 Mass spectrometry is an essential technique in the pharmaceutical field, being of the utmost
34 importance in several areas of product discovery, formulation, analysis, development and
35 manufacturing. While high-resolution mass spectrometry (HRMS) is required for accurate
36 structural elucidation, low-resolution mass spectrometry (LRMS) is useful in routine analysis
37 applications as a support to pharmaceutical process chemistry, drug discovery and early
38 development. Notably, LRMS analyzers are prone to miniaturization and thus more inclined
39 to serve the need of having a “benchtop size” MS system. In this context, a comprehensive
40 list of compact mass spectrometers was recently reported.^{153,154} Among others, compact MS
41 systems suitable for direct coupling to chromatographic systems for benchtop use included
42 the single quadrupole mass analyzers 4500 MiD from Microsaic Systems,¹⁵⁵ the Expression
43 series from Advion,¹⁵⁶ and the ACQUITY QDa Detector from Waters.¹⁵⁷ The above-
44 mentioned MS detectors are all equipped with an ESI source, allowing positive and negative
45 ionization modes in sequential analysis (4500 MiD and Expression) or the ability to cycle
46 through four different acquisition modes within a single run (ACQUITY QDa).¹⁵⁴ Full scan MS
47 or selected ion monitoring (SIM) can be selected as acquisition modes with an average mass
48 range covering 40-1280 m/z and the ability to uphold LC flow rates up to 500 $\mu\text{L}/\text{min}$
49 (Expression), 1000 $\mu\text{L}/\text{min}$ (ACQUITY QDa), and 2000 $\mu\text{L}/\text{min}$ (4500 MiD).
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The possibility of fitting MS into the chromatography workflow at the benchtop level has

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3 represented a real breakthrough in separation science, finding many applications especially
4 in the field of pharmaceutical analysis. As an example, compact benchtop MS systems can
5 be used for reaction monitoring as support to pharmaceutical process chemistry and drug
6 discovery. In fact, the presence of reaction starting materials, products and intermediates can
7 be simply followed at unit mass resolution in either the full scan or SIM mode. Some
8 examples reported in the literature include the monitoring of salicylic acid formation, the
9 quantitative analysis of phenylalanine, tyrosine, tryptophan and kynurenine in a rat model for
10 tauopathies¹⁵⁸ and the monitoring of cross-coupling reactions.¹⁵⁹

11 Another application of LC-MS analysis involving compact and affordable MS analyzers might
12 involve the identification and quantification of active pharmaceutical ingredients (APIs) and
13 their degradation products or trace impurities, as reported for the analysis of a mixture of
14 loratadine,¹⁶⁰ where impurities at low levels (0.1%) were identified with adequate sensitivity
15 by a compact Advion MS analyzer. In another configuration, consisting of a SFC system
16 coupled to an ACQUITY QDa, the quantitative determination of panthenol (provitamin B5)
17 enantiomers in cosmetic formulations was achieved with a limit of quantification (LOQ) of 0.5
18 $\mu\text{g/mL}$, with full validation of the method in terms of the linearity, precision and accuracy.¹⁶¹ In
19 another contribution, the quantitative determination of degradation products and process-
20 related impurities of daclatasvir in a pharmaceutical dosage form was performed through a
21 UHPLC-PDA/QDa workflow after hydrolysis, oxidative, photolytic and thermal stress
22 conditions.¹⁶² In addition, the method was validated in terms of its specificity, precision,
23 linearity, accuracy, LOD, LOQ and robustness according to ICH guidelines.

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

32 Applications for biotherapeutics analysis are also feasible if peptides and proteins are of
33 small to moderate sizes (up to a maximum of 6 kDa). In fact, identification is possible within
34 this size range because the ESI ionization process generates multiply charged ions that bring
35 the m/z values into the mass range of the simple quadrupole mass analyzer, therefore
36 allowing their detection that would be otherwise hampered for the molecular ion alone. In this
37 context, the latest contributions show the feasibility of UHPLC-QDa workflows for the
38 analysis of peptides (angiotensin II, ~1 kDa) and small proteins (insulin, ~5 kDa), with
39 detection and quantitation at the femtomole level.¹⁵⁴ In addition, the improved sensitivity and
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3 specificity for identity and purity testing of intact insulin, gained through the addition of
4 compact MS detection, was discussed by Zhang *et al.*¹⁶⁵ In this specific workflow consisting
5 of LC-UV/QDa, the addition of MS detection also enabled the analysis of low abundance
6 impurities.
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9 Finally, the potential application of compact mass detectors in open access (OA)
10 environments or in routine pharmaceutical quality control (QC) environments has already
11 stimulated the imagination and curiosity of several research groups. In fact, after overcoming
12 the factors limiting the widespread use of high-end MS instruments (high purchasing costs,
13 expensive maintenance, and need for highly skilled operators for method optimization and
14 subsequent data interpretation), the use of MS in OA or QC environments can be concretely
15 considered a reality.
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18 In a contribution by Gao *et al.*,¹⁵⁹ the suitability of compact mass detectors as potential OA
19 LC-MS platforms in the drug discovery and early development space was discussed. The
20 benefits of compact mass detectors were demonstrated in a wide variety of OA applications,
21 such as standard small molecule analysis, reaction monitoring, purity assessment, high
22 throughput screening (HTS) and QC-type analyses as well as in bioanalysis. Interestingly,
23 mass data generated with the compact mass detector were comparable to conventional
24 mass spectrometer data for a multitude of samples run in the OA environment.¹⁵⁹
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27 Similarly, D'Hondt *et al.*¹⁶⁶ investigated the possibility of implementing compact MS detectors
28 in existing HPLC/UHPLC equipment and software platforms used in QC. A UHPLC-UV/MS
29 equipment set-up was evaluated in relation to a traditional HPLC-UV set-up for the QC and
30 impurity profiling of complex therapeutic peptides, namely the analysis of bleomycin sulfate,
31 tyrothricin, vancomycin HCl and bacitracin peptide APIs. As expected, the UHPLC separation
32 resulted in a higher resolution and a lower limit of detection as well as a significant reduction
33 in the run time. Furthermore, the MS detector enabled the direct identification of impurities
34 and components, even at low levels, without the need for reference standards.¹⁶⁶
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46 **8. Process analytical technology**

47 Process analytical technology (PAT) has been defined as a mechanism to design, analyze
48 and control pharmaceutical manufacturing processes through timely measurements of critical
49 quality attributes during processing.¹⁶⁷ Thanks to PAT, errors can be corrected while the
50 process is in progress so that material losses can be avoided or strongly reduced during an
51 industrial process. Applying this PAT approach has the ability to save the time and money
52 required for product sampling and analysis and to produce quality and stable products in a
53 shorter period of time.¹⁶⁸
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3 Many of the progresses in continuous manufacturing are closely related to the Food and
4 Drug Administration (FDA) Pharmaceutical Quality for the 21st Century Initiative, which aims
5 at promoting the modernization of pharmaceutical manufacturing thanks to the
6 implementation of quality by design (QbD) and PAT to enable new manufacturing
7 technologies.
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11 Therefore, there have been many attempts in the last few years to integrate PAT and
12 continuous manufacturing within pharmaceutical industries.¹⁶⁹ In many cases, spectroscopic-
13 based tools such as ultraviolet-visible (UV-vis), near-infrared (NIR), Fourier transform
14 infrared (FT-IR), and Raman spectroscopy have been successfully used for real time
15 monitoring and process control,¹⁷⁰ including the monitoring of extraction and crystallization
16 processes, the production of solid dosage forms, and the quantitation of active substances
17 and impurities.¹⁶⁸ However, these approaches often lack specificity and sensitivity for several
18 other PAT applications. For this reason, chromatography might be considered to be a useful
19 strategy considering its excellent robustness, specificity and resolution. As recently reported,
20 the implementation of on-line HPLC typically includes three steps: (i) a mechanism for
21 automated sampling, (ii) sample pretreatment such as dilution, and finally (iii) the actual
22 chromatographic and data analysis.¹⁷⁰
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31 On-line LC for process monitoring has been previously demonstrated in the pharmaceutical
32 industry, but the instruments were often customized designs to support only one given
33 process. Such an instrumental setup, where a traditional off-line HPLC has been transformed
34 for on-line analysis, was recently proposed by Tiwari *et al.*¹⁷⁰ In this study, the authors have
35 used a 2 way/6 port valve to facilitate the simultaneous automated sampling of product
36 stream elution from a process column and fractionation. In the proposed methodology, no
37 sample dilution was required. A fast HPLC separation was performed, and the versatility of
38 the system has been demonstrated through the monitoring of two of the most common
39 separations required during the production of mAbs, namely the separation of charge
40 variants and aggregates. This study demonstrates that the proposed on-line HPLC
41 configuration can be used for PAT applications in preparative chromatography to facilitate
42 real-time decision-making. As a possible continuation of their work, the authors also
43 mentioned that the proposed setup could also be integrated with UHPLC to further increase
44 productivity (faster analysis times).
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54 Despite this successful example a customized on-line LC system, such a technical solution
55 often remains expensive to develop, and above all, its flexibility and portability remain clearly
56 insufficient. Recently, commercial on-line LC instruments have been introduced (e.g.,
57 Waters PATROL UPLC Process Analysis System) offering an integrated and more robust
58 PAT approach.¹⁷¹ Patel *et al.* have recently highlighted the possibilities offered by such a
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3 commercial system for on-line LC as a PAT tool for controlling mAb fermentation and
4 purification processes through the continuous monitoring of biological processes and forced
5 degradation studies. This work focused on ion exchange chromatography for the detection of
6 charge variants. First, it is important to note that no significant differences were observed in
7 the mAb charge heterogeneity profile with at-line and on-line sampling. The on-line method
8 also had the ability to rapidly detect changes in protein quality over time. The robustness and
9 versatility of the PAT methods were tested by sampling from two purification locations in a
10 continuous mAb process, demonstrating that the distribution of acidic, main, and basic
11 species percentages remains unchanged over a period of two weeks. Then, a forced
12 degradation study showed an increase in acidic species and a decrease in basic species
13 when sampled on-line over 7 days. According to the authors, implementation of on-line IEX
14 also has the potential to be applied to biomanufacturing.¹⁷¹

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16 Last but not least, a similar PAT instrument was also recently applied as an on-line tool for
17 the crystallization of three active pharmaceutical ingredients (i.e., caffeine, acetylsalicylic acid
18 and a patented drug from GSK). Indeed, concentration is an important process parameter in
19 pharmaceutical crystallization processes, and the real-time multicomponent concentration
20 was monitored in pharmaceutical crystallization¹⁷² with PAT technology.

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

24 25 26 27 28 29 30 31 32 33 34 35 36 37 **9. Automated tools for method development in chromatography**

38 Method development in HPLC intends to obtain the optimal chromatographic operating
39 conditions (i.e., types of mobile and stationary phases, temperature, gradient steepness, pH,
40 ionic strength, etc.) resulting in the required separation of a mixture of compounds into its
41 constituents within a reasonable analysis time.¹⁷³ The method development process is often
42 tedious and time-consuming (up to several weeks of work). It requires knowledge and
43 expertise from the analyst and still involves many trial and error processes. However,
44 computer-assisted and automated method development tools have the potential to speed up
45 this process. Systematic method development typically involves two phases, namely the
46 scouting and optimization phases. Structure-retention databases are of interest to speed up
47 the scouting phase and could potentially replace the initial exploratory experiments by
48 prediction solely based on the structures of the molecules. Besides speeding up the method
49 development process, systematic experimentation using state-of-the-art software packages
50 also allow the Quality by Design (QbD) principles required in pharmaceutical laboratories to
51 be met by providing a tool to improve the robustness of a chromatographic method eminently
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3 important for a safe production process. In addition, computer-assisted method development
4 reduces the solvent consumption by limiting the required number of experiments.
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7 To find the most appropriate stationary phase and mobile phase conditions, it might be useful
8 to initially perform a screening process (often known as “scouting”), as prior knowledge
9 influences the choices.^{164,174–176} Screening is typically performed on three or four selected
10 columns possessing different chemistries and operating at three different mobile phase pH
11 values in addition to using two different organic modifiers and performing a generic (short)
12 gradient. Then, the number of resolved peaks, peak shapes and elution windows are usually
13 compared to determine the best combination of stationary phase and mobile phase. This
14 approach has been successfully applied for a mixture of eight probe substrates and 8 CYP-
15 specific metabolites as well as for the separation of phytocannabinoids and *Cannabis sativa*
16 extracts.^{6,164}
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19 Today, several commercial software programs and associated hardware are available for
20 automating the mobile phase and column screening, including Nexera Method Scouting
21 Solutions™ from Shimadzu, ChromSwordAuto™ and ChromSword Scout™ from Thermo-
22 Fisher Scientific, AutoChrom™ from ACD labs and Fusion™ Method Development from
23 Waters.^{177,178} To utilize these software programs, certain hardware configurations (e.g.,
24 multichannel pumps and selection valves for columns) are required. The evaluation of
25 multiple mobile and stationary phases in an automated fashion reduces the method
26 development time and allows for efficient exploration of the separation parameters. As an
27 example, using the Nexera Method Scouting Solutions software, up to 96 combinations of
28 columns and mobile phase compositions can be screened in an automated way.¹⁷⁶ It is also
29 worth mentioning that column kits dedicated for method screening/development are also
30 available (e.g., Restek USLC Method Development Toolbox, Agilent Method Development
31 Kits, Ascentis and Discovery Method Development Tool Kit from Sigma-Aldrich and Waters
32 Method Development Kits). It is suggested that the initial screening be performed on short
33 narrow-bore columns (e.g., 50 x 2.1 mm) to minimize the screening time and reduce solvent
34 consumption.
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37 For the optimization phase, several HPLC modeling computer programs have been
38 developed in the last 25 years. The DryLab software was the first tool that predicts
39 chromatograms under a much wider range of experimental conditions than would ever be
40 possible to perform in a laboratory.¹⁷⁹ One can quickly and easily determine exactly how the
41 separation would behave when a “virtual chromatographer” simultaneously varies multiple
42 parameters. The main benefit of such software programs (i.e., DryLab, Osiris, ADC LC
43 simulator and AutoChrom, ChromSword Developer and ChromSword Off-line) is that by
44 using initial data generated from only 2–12 input experiments, the resolution and retention
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3 times for hundreds (up to millions) of unique virtual conditions can be calculated. As an
4 example, DryLab uses real data to create color-coded maps plotting critical resolution as a
5 function of one, two or three method parameters. In addition to visualizing the interactions of
6 these parameters, one can also predict chromatograms for changes in other method
7 conditions, such as the column dimensions, flow rate, gradient elution, instrumental
8 parameters, and many others.

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

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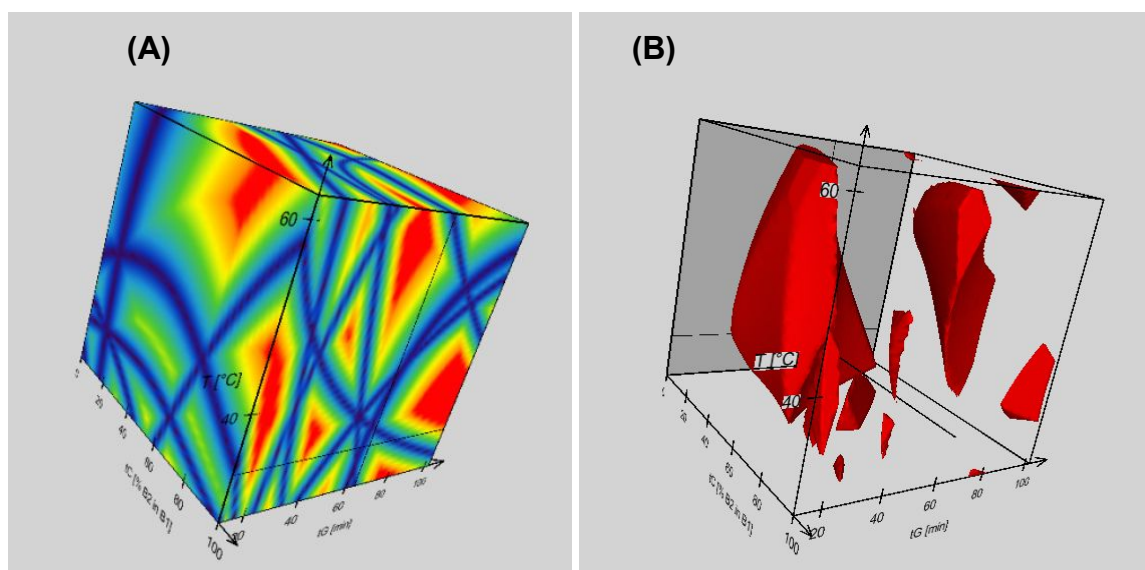


Figure 11. The illustration of a 3-D resolution cube. Baseline resolution regions are displayed in red; they are the method operable design region (MODR) visualized design space. Blue regions indicate method failure, where the critical resolution is equal to 0, which corresponds to peak overlaps (A). 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

1
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3 RPLC) for a long time. Recent works proved the applicability of retention modeling for large
4 molecules (biopharmaceuticals) in IEX, HIC and HILIC modes as well.¹⁸⁷ In contrast to small
5 molecules, large molecules such as proteins show different retention mechanisms including
6 the following: (i) an on/off mechanism retaining the macromolecules at the column inlet until
7 at some point in the gradient they are desorbed and then move through the column without
8 any further interaction; (ii) precipitation-redissolution, i.e., separation based on solubility
9 instead of interaction with the stationary phase; and (iii) multipoint attachment to the surface
10 of the stationary phase.^{173,188,189}

11 While these mechanisms are fundamentally different from those observed with small
12 molecules, the gradient separation of macromolecules can still be predicted from the linear
13 solvent strength (LSS) theory or from slightly modified models. The reason is that in most
14 cases, a relatively limited range of the method variables has to be studied because sufficient
15 retention, recovery and peak shape can only be obtained in a limited design space. The
16 other facilitation with large molecules is that generic conditions can be applied for different
17 protein classes (e.g., cytokines, mAbs, and antibody drug conjugates (ADCs)). Indeed, the
18 structures of the different proteins within a class are very similar; the amino acid sequences
19 are very close, and the global conformations are similar. It is also clear that the variants,
20 which have to be separated from the native protein and from each other, possess relatively
21 small differences compared to the native protein (such as oxidation or deamidation of some
22 amino acids or reduction of a disulfide bond). In a whole protein structure, those
23 modifications are minor compared to the native amino acid sequence (e.g., modification of 2-
24 5 amino acids from the total few hundred or thousand amino acids in the protein backbone).

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

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3 model was developed to predict the retention times for new solutes based only on their
4 chemical structures. A compound classification, based on the concept of similarity, was
5 applied prior to QSRR modeling. With the combination of QSRR and DoE approaches, a
6 workflow has been proposed to facilitate HILIC method development. Other QSRR models
7 were also developed to predict the retention times on five different HILIC phases to select the
8 most suitable column.¹⁹² To overcome the problem of HILIC retention modeling, Tyteca *et al.*
9 proposed the use of the so-called predictive elution window shifting and stretching (PEWS²)
10 approach.¹⁹³ In this computer-assisted strategy, only approximate predictions of the
11 retentions of the first and the last peaks in the chromatogram are required to conduct a well-
12 targeted trial-and-error search, with the suggested search conditions uniformly covering the
13 entire possible search and elution space. This strategy was used to optimize the separation
14 of three representative pharmaceutical mixtures possessing diverse physicochemical
15 properties. Another study showed that building models based on clustering the compounds
16 and their similarity (retention factors) appeared to be an effective approach in minimizing the
17 prediction errors in HILIC.¹⁹⁴ The concept of chromatographic similarity in QSRR could be
18 implemented by localized modeling using a measure of similarity that adequately reflects
19 solute retention. A recent review summarized the possibilities of chemometric-assisted
20 method development in HILIC.¹⁹⁵ Some recent works focused on tryptic peptide retention
21 modeling in HILIC for proteomic purposes.^{117,196,197} Those models can help with peptide
22 identification from protein digests and decrease the risk of false positive results.

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35 The other critical mode of chromatography (from the method development point of view) is
36 SFC due to the nature of the mobile phase (compressible and less viscous) and its mixed-
37 mode retention mechanism. Further complications come from the fact that practical SFC
38 mostly applies sub-critical conditions, and therefore, the mobile phase is often more liquid-
39 like or possesses a hybrid state between supercritical fluid and liquid. Therefore, the
40 temperature and pressure often work against each other and have a competitive effect on
41 the retention. Significant changes are expected in retention when slightly changing the back-
42 pressure, flow rate, mobile phase co-solvent proportion, additive concentration or mobile
43 phase temperature. For all these reasons, retention in SFC is less controllable compared to
44 LC, and retention modeling is hardly feasible. Recent works suggested different ways to
45 perform SFC method development.¹⁹⁸ As example, a generic method development approach
46 was proposed by De Klerck *et al.* to speed up the process of (UHP)SFC chiral separations of
47 drugs.¹⁹⁹ Delahaye *et al.* reported the application of stationary phase-optimized selectivity
48 predictions for isocratic SFC separations by demonstrating the applicability of this tool to
49 predict the retention of solutes on different columns under isopycnic conditions.²⁰⁰ Dispas *et al.*
50 proposed the transfer of the whole design space (DS) when sending the method to a
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3 receiving laboratory as being a useful tool to assist and speed up the commonly used
4 geometrical transfer.²⁰¹
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7 Despite the huge potential of chromatographic modeling, computer-assisted and automated
8 method development tools are still not widely used in the pharmaceutical industry. The
9 reason is probably that most QC and routine laboratories apply previously developed older
10 methods (suggested by Pharmacopeias and guidelines). Regulatory agencies and
11 pharmacopeias should promote software-assisted method development, which makes
12 studying the robustness, method transfer and method adjustment much more flexible and
13 systematic as well as less time-consuming.
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20 **10. Chiral separations**

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22 Since many drugs are chiral, pharmaceutical companies are putting increasing efforts toward
23 the development of improved and well-controlled enantioselective manufacturing processes.
24 Stereoisomers of a chiral drug can indeed differ in potency, toxicity, and behavior
25 (pharmacodynamics) within biological systems as well as in terms of their absorption,
26 distribution, metabolism, and excretion (pharmacokinetics). Inter-conversion of stereoisomers
27 is also possible for some chiral molecules due to their configurational instability around the
28 chiral centers. Racemization or epimerization can occur at any time during the
29 pharmaceutical and pharmacological time scales (development, manufacturing, storage, and
30 time spent in the body under physiological conditions).
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37 Continued discoveries of significant differences in the effectiveness and toxicity of individual
38 enantiomers in biological systems has maintained the importance of chiral separations and
39 motivated the development of new chiral stationary phases (CSPs). More than 110 CSPs
40 have been introduced during the last 15 years, but there are now a few preferred ones, which
41 satisfy the majority of chiral separation needs in the pharmaceutical industry. Cellulose and
42 amylose phases are still very popular, while the macrocyclic glycopeptide applications have
43 significantly expanded and taken on a complimentary role. Cyclodextrin derivatives and π -
44 complex Pirkle-type CSPs (e.g., Whelk-01) have fulfilled the remaining needs. A new
45 generation of immobilized polysaccharide CSPs was also introduced a few years ago. One of
46 the most successful ones is Chiralpak IC, which is a covalently bonded tris(3,5-
47 dichlorophenylcarbamate) derivative of cellulose. This phase had never been made available
48 as a coated version due to its high solubility in organic solvents. As a bonded phase, it has
49 demonstrated good selectivity for those analytes previously not resolved or poorly resolved
50 by the other immobilized cellulose and amylose derivatives. Other coated derivatives such as
51 the methylchlorophenyl carbamate of cellulose and of amylose are also available today.
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3 These phases were originally developed on 20 μm silica particles for preparative applications
4 only, but they are now available with smaller particle sizes for analytical applications.
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7 In the last decade, the attention of column developers has moved from the research of novel
8 CSPs to the preparation of new versions of already known CSPs prepared on high
9 performance silica particles, such as sub-2 μm FPPs and sub-3 μm SPPs.²⁰² Based on the
10 needs from pharmaceutical industries, e.g., for the screening of large libraries of chiral
11 molecules or for high-throughput analysis, providers have developed columns for faster and
12 more efficient chiral separations, which could not be achieved earlier on conventional CSPs.
13 Pirkle-type (often referred to as brush-type) CSPs were the first ones employed for the
14 preparation of sub-2 μm chiral particles.²⁰³ The first Pirkle-type UHPLC CSP was prepared in
15 2010 (FPPs of 1.9 μm with a DACH-DNB selector). The kinetic performance of columns
16 packed with this CSP was compared to those of columns packed with 4.3 and 2.6 μm
17 particles. The sub-2 μm column displayed both a significant efficiency gain and reduced
18 analysis times, while maintaining comparable selectivity and improving the resolution.
19 Barhate *et al.* functionalized narrow particle size distribution particles (1.9 μm) with
20 macrocyclic glycopeptide selectors.²⁰⁴ Sub-minute separations of amino acids, β -blockers
21 and heterocyclic compounds of pharmaceutical interest were demonstrated. A new synthetic
22 process was recently reported to produce the zwitterionic version of teicoplanin sub-2 μm
23 CSP and applied to separate the enantiomers of amino acid derivatives of polar acidic
24 compounds. It also showed large selectivity for organic/inorganic ions.^{205,206} To further speed
25 up chiral separation, Gasparini *et al.* combined the use of a short column (1 cm length) and
26 high flow rates (8.0 mL/min). Figure 12 shows an example of such an ultra-fast approach.
27 For the sake of completeness, some other chiral UHPLC fast separations are reported in a
28 recent review.²⁰⁷

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

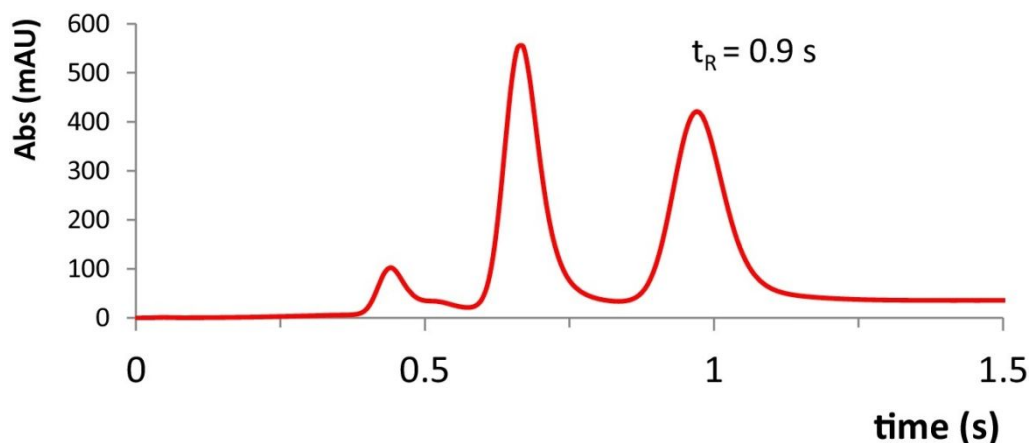


Figure 12. Example of ultrafast enantioseparation (enantiomers of trans-stilbene oxide) thanks to the use of a short column and high flow rate. A 10×3.0 mm column was operated at 8 mL/min. Reprinted from *J. Chromatogr. A*, Vol. 1466, Ismail, O. H.; Pasti, L.; Ciogli, A.; Villani, C.; Kocergin, J.; Anderson, 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 demonstrated²¹⁶ 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 phenomenon²²⁰ 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 enantioselectivity. 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 \times chiral and chiral \times 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 \times chiral 2D-LC method for the complete resolution of isomers of a synthetic intermediate.

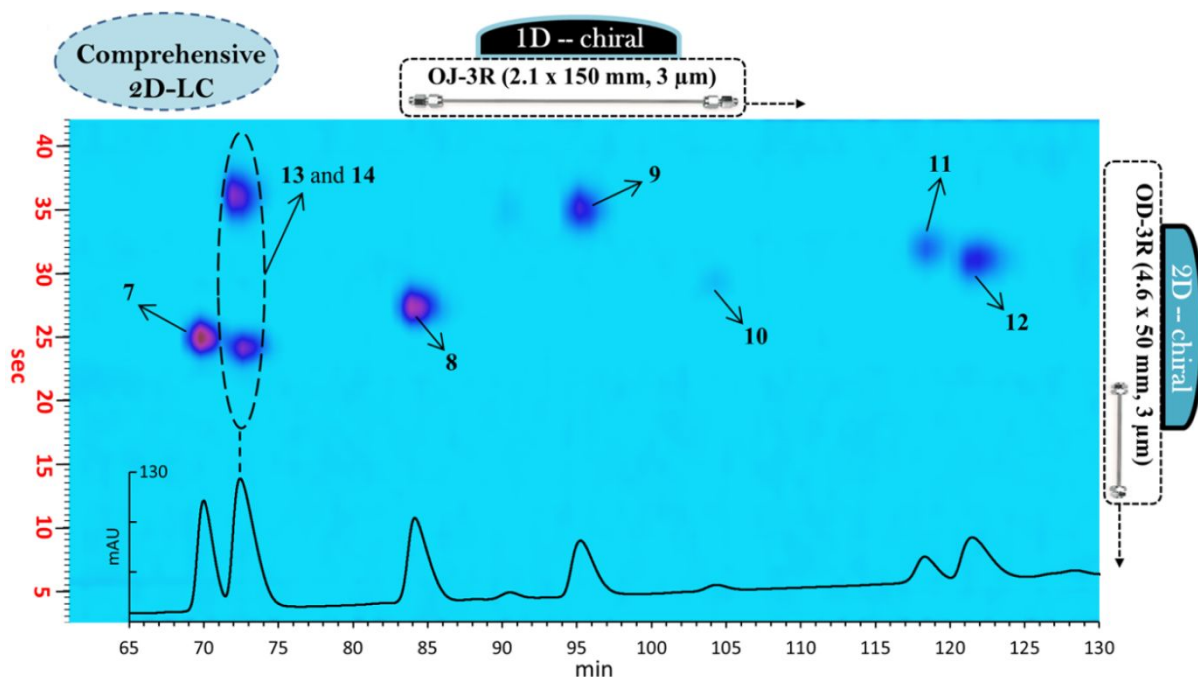


Figure 13. Comprehensive chiral \times chiral 2D-LC method for the complete resolution of isomers of a synthetic intermediate. Conditions, first dimension (chiral): column, OJ-3R column (2.1 mm \times 150 mm, 3 μm); flow rate, 0.05 mL/min; mobile phase, eluent A, 0.1% H_3PO_4 in H_2O and eluent B, ACN/MeOH (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 \times 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 $\mu\text{g/g}$) but that may well fall between 10 and 1000 ppb (10 ng/g and 1 $\mu\text{g/g}$, 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

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3 discover the most suitable analytical method.^{229–231} Based on the volatility of the
4 PGIs/GIs/MIs, the selection of its analytical technique can lead to either gas chromatography
5 (GC) or liquid chromatography (LC) analysis, whereas the choice of the detector is defined
6 based on the required limits the impurity must meet while avoiding matrix effects.
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10 For volatile PGIs/GIs/MIs, GC with flame ionization detection (FID) is generally applied as a
11 first choice. Then, if the desired sensitivity is not obtained and the method cannot be finalized
12 and validated, either other detectors are evaluated or the volatility and stability of the
13 PGIs/GIs/MIs is improved through derivatization or extraction approaches to minimize the
14 matrix effects. 2D-GC approaches can be applied as a last resort when all of the above-
15 mentioned techniques were unsuccessful in terms of selectivity.^{229,230}
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19 As an example, a sensitive and reliable GC–MS method was developed, optimized and
20 validated for the simultaneous determination of 4 closely related GIs, namely 3-chloro-1-
21 propanol (CHP), 1,3-dichloropropane (DCP), 3-chloropropylacetate (CPA) and chloropropyl
22 hydroxypropyl ether (CHE), contained in the drug fudosteine.²³² Analytes were extracted in
23 dichloromethane and monitored by gas chromatography-electron ionization-mass
24 spectrometry (GC-EI-MS) in the selective ion monitoring (SIM) mode. The LODs and LOQs
25 established for CHP, DCP, CPA and CHE were in the range of 50-80 ppb (0.05-0.08 µg/mL)
26 and 100-170 ppb (0.10–0.17 µg/mL), respectively. Satisfactory separation, detection, and
27 quantitation were achieved, and the method was assessed by evaluating the specificity,
28 precision, sensitivity, linearity and accuracy.²³²
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32 For non-volatile PGIs/GIs, RPLC is generally applied as the first choice coupled to either UV
33 or MS detection depending on the chromophoric properties of the impurity. Then, other
34 modes of chromatography might be selected for polar or ionizable compounds, namely HILIC
35 and mixed-mode chromatography (MMC). Finally, 2D-LC(-MS) can be helpful when all of the
36 other approaches do not meet the required selectivity and sensitivity.^{229,230}
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40 A comprehensive example of method development for PGI identification and characterization
41 was recently published by Huang *et al.*²³³ In this contribution, an unknown degradation
42 product (impurity I) was detected during the stress testing of linagliptin, and it was
43 subsequently isolated, identified, and structurally characterized by orthogonal analytical
44 techniques (i.e., MS, MS/MS, 1D and 2D NMR spectroscopy, and IR spectroscopy). The
45 degradation product (impurity I) and another process-related impurity (impurity II) of
46 linagliptin were found to contain the structural alerts of N-acylated aminoaryl and alkyl halide,
47 respectively, which are both PGI substances. Based on the TTC, the permitted level of these
48 PGIs in linagliptin was 300 ppm. Therefore, an RPLC-UV method was purposely developed
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3 for the simultaneous determination of these two PGIs by reaching LODs of 20 and 10 ppm
4 and LOQs of 60 and 30 ppm for impurity I and impurity II, respectively.²³²

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

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

32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 **12. Cleaning validation**

49 Documented equipment cleaning is required to establish the cleanliness of manufacturing
50 equipment (production line) before its subsequent release for use in the production of
51 intermediates and active pharmaceutical ingredients (APIs).²³⁶ Non-dedicated equipment
52 should be cleaned at product change to prevent cross-contamination for subsequent batches
53 manufactured on the same equipment.²³⁷ Cleaning procedures should contain sufficient
54 details to enable operators to clean each type of equipment in a reproducible and effective
55 manner, and these procedures should include a complete description of the methods and
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3 materials, including the dilution of the cleaning agents used to clean the equipment. From an
4 analytical point of view, cleaning verification/validation requires the development of validated
5 methods, which are able to determine either (i) the residual APIs from the previous batch or
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7 (ii) the residual detergents, which are used to remove the drug product residues from the
8 manufacturing equipment.
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11 The basic principle of equipment cleaning is that the patient should not take more than 0.1%
12 of the standard therapeutic dose (effective dose) of a previously manufactured product.
13 Therefore, the maximum allowable carry-over (MAC or MACO) depends on the minimal daily
14 dose (active weight) of the previous product, the smallest batch size of the subsequent
15 product and the maximum daily dose (product weight) of the following product. An additional
16 criterion is the 10 ppm limit, which suggests that not more than 10 ppm of the previously
17 manufactured product is allowed to appear in the subsequent product. If the MAC value
18 becomes greater than 10 ppm, then the 10 ppm criterion needs to be considered. For
19 detergents, as many of them are considered to be slightly or moderately toxic based on their
20 LD₅₀ data, their carry-over is acceptable up to a 100 ppm limit. The determination of both the
21 residual API and detergent requires very sensitive methods. Therefore, the assessment of
22 residual APIs and detergents at the end of the equipment cleaning process is challenging
23 because the analytical method has to address the limits of quantitation at trace levels (e.g.,
24 ppm) of analytes that often lack chromophores.
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27 Analytical methods used to determine the residuals of APIs should be specific for the
28 substance or class of substances to be assayed and be validated prior to cleaning validation.
29 HPLC–MS and UHPLC-MS techniques applied in pharmaceutical cleaning verification have
30 the advantages of improved sensitivity, selectivity and general applicability, even for UV-
31 inactive compounds. However, these techniques are quite expensive and still not widespread
32 in cleaning control analysis. The current trend seems to develop methods not only for a given
33 API but in a more generic way. The concept of applying a generic method for several API
34 residues for a product line was found to be feasible and practical if the structures and
35 properties of the compounds to be determined are similar. For such a purpose, a generic
36 UHPLC-UV method has been reported for the fast determination (analysis time of 2.5
37 minutes) of various steroid residues in support of cleaning validation. Another study
38 described the development of a single 10 minute gradient HPLC/UV method for cleaning
39 verification applications at 0.2 to 10 µg/mL levels for many APIs.²³⁸ Additional sensitivity
40 enhancement can be accomplished by large volume injections, sample enrichment and the
41 use of long-path UV flow-cells. The general adoption of generic platform technologies can
42 greatly enhance analytical laboratory productivity by simplifying lab operation and reducing
43 method development and validation efforts. This standard method is readily adaptable to
44 swabbing or rinse solutions.
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3 For detergents containing chromophore groups, the HPLC-UV method is suitable for their
4 detection and quantitation in most cases. If a chromophore is absent, other techniques such
5 as ion chromatography with conductivity detection or charged aerosol detection can be
6 used.²³⁹
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9 The most critical parameter of the analytical methods applied for cleaning validation
10 processes is the correct sampling and related solute recovery. The surface of the equipment-
11 line consists of mostly (> 95%) stainless steel, but there are other critical surfaces, which are
12 made of Plexiglas, polytetrafluorethylene, silicone (gaskets) and textiles. These specific
13 surfaces are hard to clean, and thus, it is necessary to sample these areas during the
14 cleaning verification/validation process (as they represent the “worst case” places of the
15 equipment). During analytical method development and validation, the solute recovery from
16 such surfaces needs to be checked. Sampling can be done either by swabbing (wiping) or
17 rinsing, depending on the accessibility of the equipment part. Typically, model coupons
18 (made of 10 x 10 cm stainless steel or other representative materials) are used for recovery
19 studies. The API or detergent is added (spiked) in a known concentration onto the surface
20 and then wiped using a wetted swab. The content is then extracted with an appropriate
21 solvent. The parameters affecting the recovery of APIs from the surfaces of stainless steel
22 coupons have been recently studied, including the API level, spiking procedure,
23 API/excipient ratio, analyst-to-analyst variability, inter-day variability and cleaning procedure
24 of the coupons.²³⁷ The lack of a well-defined procedure that consistently cleaned the coupon
25 surfaces was identified as the major contributor to low and variable recoveries. The
26 assessment of acidic, basic and oxidant washes as well as the order of treatment, showed
27 that a base-water-acid-water-oxidizer-water wash procedure resulted in a consistent and
28 accurate spiked recovery (> 90%) as well as reproducible results. An interesting feature of
29 such a modeling of API recovery was also pointed out, namely that the surface of the coupon
30 may be modified during its use due to the deposition of a thin film of material on the surface,
31 which may result in altering the oxidation states of the metals on the surface. Therefore, a
32 time-dependent decrease in recovery is expected due to binding between the metals and the
33 analyte of interest. Indeed, the repeatability of sampling decreased with an increasing
34 number of replicates, and therefore, the regeneration of coupon surfaces seems to be
35 mandatory. Another study showed that a good correlation could be found between the
36 recoveries of solutes and their retention factors measured in RPLC. More hydrophobic
37 solutes showed worse recoveries in most cases. Silicone rubber surfaces were found to be
38 the most critical surfaces during the sampling process. It is recommended to apply these
39 silicone gaskets as dedicated parts of the equipment.
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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 Anastas²⁴³ 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 $^{\circ}\text{C}$), only pure water can be used as a mobile phase. This strategy

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

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12 The next strategy for greening LC is to use greener mobile phase components. In particular,
13 acetonitrile and methanol have been replaced by acetone or ethanol, which can both be
14 considered to be more environmentally friendly mobile phases. Ethanol is particularly
15 desirable since it has close properties to methanol but is less volatile, less toxic and has
16 lower disposal costs.^{249,250} However, there are three chief barriers to the replacement of
17 acetonitrile with ethanol, namely, the high viscosity, substantially different chromatographic
18 selectivity, and restrictions in trading. To avoid this latest issue, some people from the
19 pharmaceutical industry have suggested performing chromatography with distilled alcohol
20 spirits (i.e., cachaça, rum, vodka, aguardiente and grain alcohol). It is indeed available at a
21 relatively inexpensive price from local markets in virtually all regions of the world, offering the
22 possibility of local sustainable production of a green nontoxic eluent for HPLC. This strategy
23 was named “cocktail chromatography” and offers, in many cases, excellent analytical
24 performance.²⁵¹ However, such an approach cannot be applied in the pharmaceutical
25 industry. Acetone is another alternative, but it is scarcely used as a mobile phase component
26 since it is a strong UV absorber up to 340 nm, which makes it impractical when UV detection
27 is used.²⁴⁵ Replacing organic solvents with carbon dioxide under supercritical conditions is
28 another powerful approach to make LC greener,²⁵² since it is characterized by non-toxicity,
29 non-flammability, low disposal costs and above all limited environmental impact (see Section
30 4 on SFC). In HILIC separations, the addition of CO₂ to the conventional mobile phases
31 makes the replacement of acetonitrile with methanol or ethanol possible, resulting in greener
32 separations. This strategy has been recently applied for the analysis of
33 nucleosides/nucleotides,²⁵³ amino acids,²⁵⁴ drugs and natural products.²⁵⁴

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48 The last strategy to minimize organic solvent consumption is to play with the column-related
49 parameters. Indeed, when reducing the column internal diameter, the flow rate has to be
50 scaled down by the square of the column diameter without affecting the analysis time as well
51 as peak width and provided that the extra-column volume remains acceptable. Moving from
52 the classical 4.6 mm to 3 mm I.D. leads to a 57% reduction in the volume of solvent and
53 waste. Today, the use of narrow-bore (2.1 mm I.D.) columns is becoming increasingly
54 popular in the pharmaceutical industry due to the significant progress in chromatographic
55 instrumentation over the last few years (UHPLC instruments with much lower extra-column
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3 volumes). Micro-bore (1 mm I.D.) and capillary (0.3 mm I.D.) LC columns constitute the
4 ultimate approach in this direction but require specific instruments,²⁴⁶ which are not yet
5 widespread in the pharmaceutical industry.
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10 **14. What are the latest advances in GC?**

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12 Despite Gas Chromatography (GC) predating HPLC, it still remains a lesser used technique
13 in pharmaceutical analysis owing to the insufficient volatility and thermal stability of the
14 majority of APIs. Nevertheless, GC and GC-MS remain essential tools in qualitative and
15 quantitative pharmaceutical analysis, including: residual solvents in APIs and pharmaceutical
16 products and ingredients; volatile organic compounds (VOCs) such as toxic and genotoxic
17 impurities and degradation products (see Section 11); extractables and leachables; and also
18 the analysis of raw materials, (e.g. solvents and reagents) and starting materials/synthetic
19 building blocks (e.g. impurity profiling and characterization). When pharmaceutical analytes
20 are amenable to GC, it is often the technique of choice as it has multiple advantages over
21 HPLC, owing to its high efficiency and resolving power, simple operation, and a universal-like
22 response and wide dynamic range of the flame ionisation detector (FID).
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30 Fast GC using narrow-bore/micro-bore columns and fast temperature programming rates
31 was developed to decrease analysis times. This was followed by ultra-fast GC (UFGC) using
32 resistively heated temperature programming with ballistic heating rates, which lead to faster
33 analysis times (2-20 times), thus reducing power and also carrier and detector gas
34 consumption.^{255,256} Fast GC/Ultra-Fast GC has been successfully applied in pharmaceutical
35 applications. However, despite these advantages, the uptake of these technologies in the
36 pharmaceutical industry was limited owing to perceived poor usability, fragility/poor
37 robustness, and higher costs. Recent enhanced commercial instruments (e.g. Agilent
38 Intuvo™) have renewed interest in UFGC owing to their reduced footprint and improved
39 workflows and usability via features such as simple or ferrule free connections, ease of
40 changing guard and analytical columns, improved serviceability/reduced maintenance, and
41 improved user interfaces etc.^{256,257}
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49 In recent years, there has been several workflow-based innovations related to increasing
50 productivity *via* decreasing overall analysis times using existing technology, but with
51 alternative methods and ways of working. Scientists from most Pharmaceutical R&D groups
52 have developed generic methods for static headspace GC for the determination of residual
53 solvents in APIs and drug products.²⁵⁸ Several R&D groups have also developed and
54 implemented open access GC and GC-MS systems using generic methods, and also
55 automated method development screening systems with columns offering different
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3 selectivity, in dual column or parallel column formats. These include systems for impurity
4 profiling and chiral analysis of volatile raw and starting materials and chemical intermediates.
5 Additionally, various generic GC-FID relative response factor (RRF) methods have been
6 developed and employed with either direct injection or headspace sample introduction. Here,
7 pre-determined RRFs against an internal standard (e.g. Decane) were determined and
8 utilised to quantify a wide range of residual solvents of interest. These approaches based on
9 lean sigma principles significantly improved the laboratory efficiency and are greener, as they
10 significantly reduce the use of sample solvents compared to external standard methods.
11 They have also been routinely employed in regulated environments (e.g. GMP).^{259–261}

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13 Several GC chromatographic modelling and method translator software are commercially or
14 freely available. One such on-line modelling program called Pro-EZGC™ simulates
15 separations using database information containing retention models of 100s of common
16 volatile organic solvents and reagents on multiple columns, and provides GC conditions and
17 column selection for analysis within ca. 5-10 minutes. Many method translators facilitate
18 rapid conversion of conventional capillary GC methods to fast or ultra-fast conditions, or the
19 evaluation of a change of carrier (e.g. He to H₂) and propose operating conditions whilst
20 maintaining chromatographic resolution, or it can propose higher speed separations with a
21 reduction in peak capacity and chromatography resolution. In the pharmaceutical industrial
22 environment, these tools are very helpful and lead to significant time savings in method
23 development and optimisation.

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25 Multidimensional GC has had continued development. Previously, although it was
26 recognised as a powerful technique, it was seen as an academic or research only tool. Other
27 users often shied away from the technology, owing to the perceived high costs and difficulty
28 to implement routinely in industrial laboratories. However, thanks to more affordable and
29 flexible, modular hardware, along with simpler workflows, data processing and databases, it
30 is now starting to be adopted in several industries by high-throughput laboratories and for
31 comprehensive screening. In particular, multidimensional GC with multiple column and
32 detection (e.g. dual detection MS and FID) has been shown to be a powerful technique in
33 regulated areas of the fragrances and flavours industries.^{262–264} Therefore, multidimensional
34 GC is also transcending into pharmaceutical analysis, but one can expect less demand and
35 utilisation owing to lower numbers of volatile analytes and the lower complexity of
36 pharmaceutical samples. Trace analysis of Mutagenic impurities is one area where 2D-GC
37 has been successfully applied owing to the improved selectivity (see section 11).^{229,230,264}
38 Metabolomics is another area where it has been utilised to support drug discovery and
39 development. Several groups are developing new technologies and strategies for the
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3 definition of the composition of volatile and non-volatile biologically active secondary
4 metabolites of plants and plant products of interest in the pharmaceutical sectors.^{265–267}
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7 Recent advances in column technology have focused on improved usability with newer
8 commercial stationary phases demonstrating higher inertness, lower bleed and higher
9 maximum operating temperatures leading to reduced maintenance and conditioning times.
10 These types of phases based on advances in polymer development and inert/deactivated
11 surfaces have improved usability and reliability in routine GC operations and may further
12 extend the interest in fast GC, specifically if there are high temperature stable and inert
13 stationary phases available in UFGC format. The development and introduction of novel
14 stationary phases is also needed for multidimensional and will further increase the use in the
15 pharmaceutical industry as well as other industries.
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19 Ionic liquid (IL) phases have unique selectivity and chromatographic properties, and have
20 been a recent addition to the range of commercial available stationary phases and offer
21 opportunities for extending the selectivity range and temperature-operating range compared
22 to conventional columns.²⁶⁸ Armstrong *et al.* have developed fully water-compatible ionic-
23 liquid IL-based capillary GC columns based on derivatives of phosphonium or imidazolium
24 cations combined with the trifluoromethanesulphonate anion. These IL-derivatives are stable
25 to water and thus it be used as injection solvent, and is thus advantageous for water-based
26 samples which often require extensive sample preparation procedures when using
27 conventional columns. These commercially available water-compatible IL columns have
28 been successfully applied in a number of industries for direct qualitative and quantitative
29 analysis of aqueous samples. Armstrong *et al.* successfully developed sensitive IL-based
30 capillary GC methods using thermal conductivity detection (TCD) or barrier ion discharge
31 detector (BID) to determine the water content in liquid samples in pharmaceutical analysis.²⁶⁹
32 These methods provides a suitable and more sensitive, but also more expensive alternative
33 to existing Karl Fischer methodologies (e.g. when having interferences or side reactions,
34 instability, insufficient solubility or limited sample quantities or when lower detection limits are
35 needed). Further long term and wider use will demonstrate if these phases are sufficiently
36 stable and robust, which has been questioned by some scientists and suggested the
37 inertness and possibly the range of operative temperature needs to be extended when water
38 is the main sample solvent.²⁷⁰
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54 Recently, innovations in GC detector technology have included Vacuum Ultraviolet (VUV)
55 and FTIR.²⁷¹ GC-VUV spectroscopy is claimed to be an easy to use universal detection that
56 provides qualitative spectral information from 125 nm to 240 nm. This has also been recently
57 utilised for residual solvents analysis in APIs and excipients and water determination.²⁷² The
58 qualitative VUV spectral information can be useful for peak purity assessment (e.g. detect
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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.

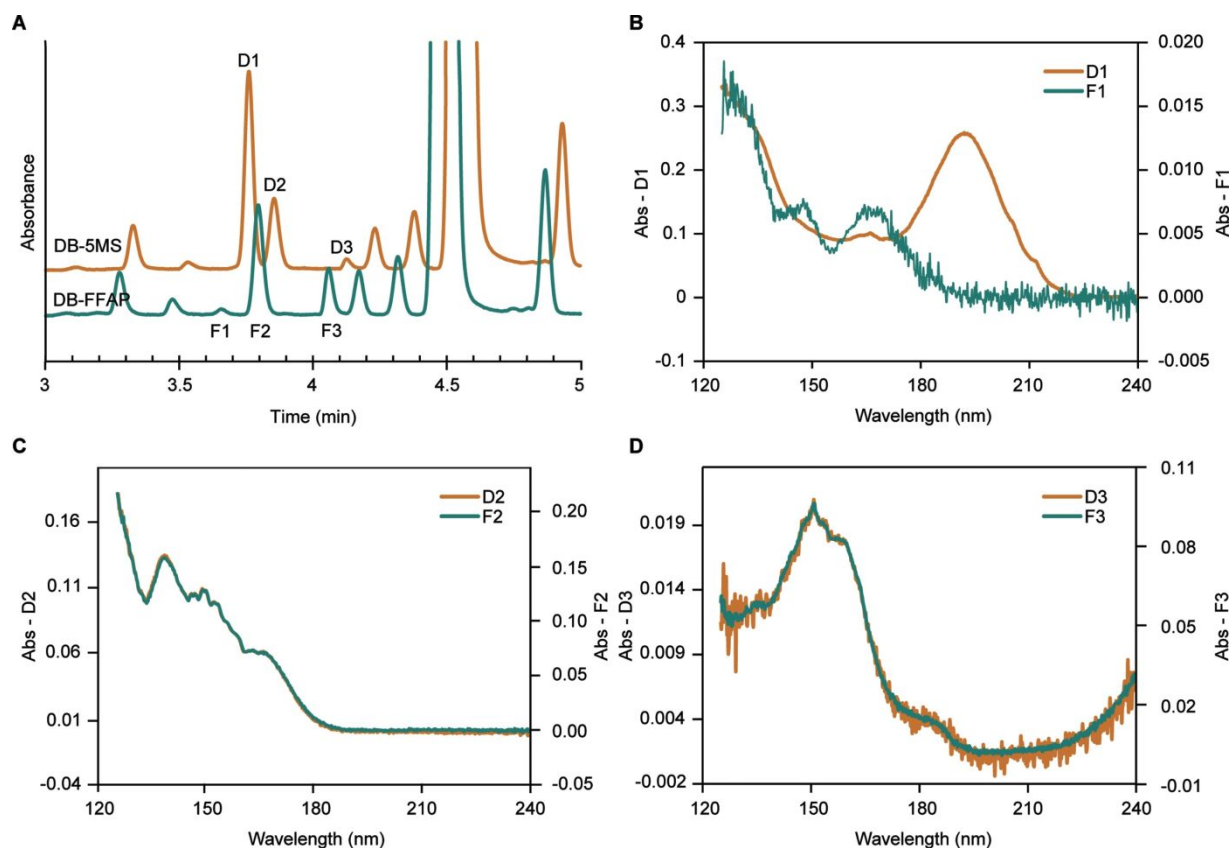


Figure 14. Peak tracking using VUV spectra. A. Overlaid chromatograms of 4-ethoxy-1,1,1-trifluoro-3-buten-2-one acquired on a DB-5MS column and a DB-FFAP column. B. VUV spectra for peak D1 and F1; C. VUV spectra for peak D2 and F2; D. VUV spectra for peak D3 and F3. Reprinted from *J. Chromatogr. A*, 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.

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3 Also GC–MS and more recently GC-HRMS play vital roles in the characterization and
4 quantitation of extractables and leachables (E&L) that may result from pharmaceutical
5 packaging materials and devices. GC-HRMS has proved to be a key technique in E&L
6 profiling using targeted (vs. references/libraries) and non-targeted screening (fully scan).
7 High chromatographic resolution, fast data acquisition, high mass accuracy combined with
8 powerful software and databases/libraries help identify and quantify impurities with higher
9 confidence. Recent work by Zdravkovic *et al.* also used GC-MS and determined relative
10 response factors (RRFs) and retention index values for over 150 diverse organic compounds
11 and generated a database, which they proposed is suitable for use in intra-laboratory
12 extractable screening studies.²⁷⁷
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19 GC and MS play an important roles in the quantitation of genotoxic impurities (GTIs) that
20 may result from devices used within the pharmaceutical industry (see section 11).^{228–232} Many
21 GTIs are volatile and have a no, or limited UV chromophore (e.g. Alkyl halides) or they can
22 be derivatized to lower the required elution temperature. Therefore, owing to the high
23 resolving power, extra sensitivity and simplicity, GC with a range of detectors (FID, ECD,
24 MS) plays an equal or more important role to LC & LC-MS. Advances in automated and
25 multipurpose sample preparation equipment (e.g. multifunctional auto-samplers, liquid
26 sample preparation, liquid-liquid extractions, Solid Phase Micro Extraction (SPME), Stir Bar
27 Sorptive Extraction (SBPE) etc.) have also facilitated the wider use of GC as part of high
28 performance and high productivity workflows in pharmaceutical analysis.
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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-MS²⁷⁹ will certainly continue its expansion.^{24,136,280}

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