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An overview of recent advances in HPLC instrumentation

Review Article

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Abstract: This review highlights the fundamentals and the most prominent advances in the field of HPLC instrumentation over the last decades. Fundamental aspects and practical considerations of column switching, conventional (heart-cut) and comprehensive two-dimensional LC are presented. Different aspects of microcolumn- and nanoliquid- chromatography are reviewed. Recent progress in column technology and the demands and developments in instrumentation and accessories for miniaturized LC are also discussed. In the field of miniaturization, particularly in chip-based nano-LC systems, some aspects on micro-fluidic chip fabrication, using particle-packed HPLC microchips or polymer-based monoliths, are addressed. An introduction to ultra performance LC (UPLC) is also presented.

Keywords: *Chip-based HPLC • Microcolumn chromatography • Multi-dimensional chromatography • Nanoliquid chromatography • UPLC*

1. Introduction

High performance Liquid Chromatography (HPLC) is one of the most popular and mature analytical techniques and by far the most widely used separation technique. It has been used in laboratories worldwide over the past 40-plus years for pharmaceutical sciences, clinical chemistry, food and environmental analyses, synthetic chemistry, *etc.* [1-5]. HPLC has gained its popularity mainly due to its reliability (use of pressure driven liquid support) and versatility (possibility of adjusting the composition of both mobile and stationary phases) [6].

The chromatographic mode or separation mechanism, depends on the overall interactive relationships between the stationary phase, the mobile phase and the analyte. Particle- packed columns with either totally porous- or the newly developed core-shell particles and monolithic columns are used in conventional or miniaturized HPLC systems.

It is the aim of this review to highlight fundamental aspects and practical considerations on: column switching, conventional (heart-cut), comprehensive two-dimensional LC and different aspects of microcolumn-, nanoliquid- and ultra performance- (UPLC) chromatography. A review of recent progress in column technology and developments in instrumentation for miniaturized LC; chip-based nano-LC systems and micro-fluidic chip fabrication, using particle-packed HPLC microchips or polymer-based monoliths are also discussed.

2. Column switching

Usually an HPLC system consists of a simple flow-through sequence of one or a few columns. If this sequence is branched and switching devices are employed to interface individual columns, it then becomes possible to exert powerful control on the quality and the basis for the separation and LC becomes a versatile and powerful separation system. This leads to the idea of column switching, which means that the carrier flow is changed by standard six-port high pressure valves, so a fraction of or all of the effluent from any primary column is selectively transferred to any one of a number of secondary columns, that form a network, for further separation. Switching within the network may be manual or automatic.

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The term column switching is used in modern LCs for different operating modes in a strictly non-defined sense. Other synonymous expressions such as “sequential chromatography”, “multiple column chromatography”, “multi-channel chromatography”, “split chromatography”, “coupled column chromatography” *etc.*, may be used for column switching, but with different objectives for chromatographic separations.

The objectives of column switching are: (1) to increase the chromatographic resolution and selectivity; (2) to enrich trace amounts of sample; (3) to protect sensitive detectors from contamination by co-extractives; (4) to prevent destabilization of the chromatographic equilibrium of the column by co-extractives; and (5) to achieve further objectives or a combination of several objectives within one chromatographic network [7-10].

There are four basic column to column transfer techniques of sample fractions, which together with three additional switching functions satisfy the needs of the majority of the on-line chromatographic separation modes such as sample clean-up and pre-concentration, chromatographic fractionation and group separation.

2.1. Transfer techniques

The transfer techniques [8] depend on the direction of the flow of the mobile phase and can be distinguished as:

(a) *Direct transfer:* The analyte fraction is separated onto the first column and is directed through the switching valve with the primary mobile phase on to the secondary column(s).

(b) *Indirect transfer*: The analytes of interest are separated by the primary mobile phase, but are not eluted from the primary column until the switching valve is rotated into the transfer position. A secondary mobile phase with a higher elution power than the primary mobile phase is directed on to the primary column transferring the analytes on to the secondary column(s).

(c) *Reversed transfer*: The analytes are not separated with the primary mobile phase on the primary column, until rotating the switching valve into the transfer position. A secondary mobile phase with equal or stronger elution power is delivered through the pump and transfers the analyte fraction, by reversing the flow direction of the primary column through the out port on to the secondary column. The secondary mobile phase now separates the analyte fraction while the primary column is reconditioned.

(d) *Loop transfer*: The primary and secondary columns are not connected on-line during the transfer period. The eluted fraction from the primary column is collected in a loop and is re-injected into the secondary column.

2.2. Switching functions

In addition to the four basic transfer techniques, three commonly used switching functions are defined [8], in order to optimize chromatographic parameters:

(a) Column backflushing is used to remove sample components that are strongly retained on the primary column and speeds up the analysis of complex mixtures without the need of gradient elution. After the fraction has eluted from the primary column and has been transferred for further separation on to the secondary column, this technique reverses the flow of the primary column to the waste.

(b) *Column selection (or column bypass)* refers to column switching systems [11-13] which are used to reduce long analysis times of analytes with very different polarities, using two or more (different) columns. Late eluting components are directed after the primary (short) column to the detector, whereas the fast eluting components are directed to the secondary (long) column, for further separation, before they are detected.

(c) *Recycling chromatography*, in comparison with the column selection technique, offers the possibility of increasing the number of theoretical plates by repeated use of the same column. A portion of the chromatographic effluent is re-injected onto the same column.

2.3. Change of chromatographic mode

The chromatographic mode or separation mechanism, depends on the overall interactive relationship between the substrate, the analyte and the carrier and is considered to be based on the column stationary phase [7]. However, the mode and the type of stationary phase are not necessarily the same. The mode control or mode sequencing comprises several changes of the composition of stationary and/or mobile phase, which contributes to overall increase in the selectivity of the system and in the separation capability [8]. The sequential changes of the chromatographic modes are known as multi-dimensional chromatography, stationary phase programming, mode switching, selectivity switching and coupled columns among other terms.

2.4. Applications of column switching

(a) *Sample clean-up* is achieved by choosing the size of the fraction to be transferred from the primary on to the secondary column, so that the fraction transferred contains as little as possible of the interfering components and as much as possible of the analyte [14,15]. By applying an on-line multi-step fractionation, *i.e.*, different switching functions and/or transfer techniques, the analyte is gradually enriched relative to interferences.

(b) *Sample preconcentration* is based on the fact that the sample components are retained in a narrow

zone on the top of the column, when a large sample volume is injected [16,17], provided that the column is not overloaded and its capacity is not exceeded. Trace enrichment is performed when aqueous solutions of relatively non-polar compounds are injected on to a reversed-phase column.

(c) *Group separation* is based on chromatographic fractionation of selected groups of components with *e.g.* similar retention characteristics or similar molecular size. Nielen *et al.* [18] used small columns packed with different stationary phases for the on-line separation and sample pre-concentration of industrial waste water. The choice of a primary chromatographic system, which selects some characteristics unique to the components being analyzed, reduces the number of components to be transferred on to the secondary column. Fewer peaks and better resolution of the secondary analytical system, compared with the direct separation of the whole sample is thus achieved.

(d) *Chromatographic adjustment* can be effected in several ways, *e.g.* by selection of a suitable column length for each analyte or selection of a suitable stationary phase for each group of analytes by the column selection technique. It can also be achieved by backflushing of the primary column for the adjustment of run time (late eluting interferences are backflushed to waste, while the analytes are separated on to the secondary column). Chromatographic adjustment by column switching is an effective alternative to solvent adjustment by gradient elution.

3. Multi - dimensional liquid chromatography

One-dimensional chromatography is widely applied to the analysis of real-world samples in several fields. However, it does not provide sufficient resolving power for the separation of complex mixtures encountered in food analysis, environmental analysis, identification of microorganisms, molecular biology, proteomics and metabolomics. A solution to this problem is to develop multi-dimensional separation schemes where the dimensions are based on different separation mechanisms [19,20].

Two-dimensional (2D) LC can be theoretically used in several multi-dimensional combinations, offering increased peak capacity, selectivity and resolution, especially in the comprehensive LC mode [19-23].

2D-LC can be performed either off-line or in an on-line mode depending on the way transfers from the primary column effluent to the second column are established. Off-line 2D-LC is the most popular approach since it is easier and very simple. Fractions of the first dimension effluent are collected manually or *via* a fraction collector, after which they are re-injected on the secondary column. However, the off-line approach can be time consuming, difficult to automate, non-reproducible and susceptible to sample loss and contamination. The on-line approach is faster and more reproducible, but it needs interfaces and is more difficult to operate [19,21].

3.1. General aspects

Some generally agreed definitions, symbols and terms that have been arrived at by mutual understanding among chromatographers are being presented here [24]. These include usage of the words “comprehensive” and “orthogonal” and of the multiplex sigh (x) as a short notation for comprehensive 2D separations. In this respect, on-line 2D-LC can be divided into “heart-cutting” and “comprehensive LC”, abbreviated as LC-LC and LC x LC respectively. In both approaches, the columns are connected *via* an interface. The main difference between these two techniques is the amount of the primary column effluent that is transferred. In heart-cutting 2D-LC, only fractions of the effluent, containing the analytes of interest are directed from the primary to the secondary column (dimension). In comprehensive LC, the entire (comprehensive) sample is subjected to separation in both dimensions. Other requirements of a comprehensive 2D separation are that any two components separated in the first dimension must remain separated in the second dimension and that elution profiles from both dimensions are preserved [24,25].

In separation science, one of the terms most inconsistently used on 2D-LC is “orthogonal”. A 2D separation is orthogonal if the two separation mechanisms are independent of each other and have different retention profiles or in other words, provide different selectivities. The two mechanisms must spread the sample components following two different retention patterns, over as broad a range possible for retention factors, so that broad separation spaces be used in both separation dimensions. The retention parameters employed in the second dimension must separate the components that the mechanism used in the first column elutes closely [20,23,26-28]. Successful orthogonal separations can be achieved when suitable mobile and stationary phases are selected, taking into account the physicochemical properties of the sample components including size and charge, hydrophobicity and polarity. Two typical examples of orthogonal combinations are the silver ion (SI) LC x non-aqueous (NA) RP-LC of triacylglycerols (separation according to degree of saturation and partition number) [29], and the NP-LC x

RP-LC separation of essential oils, containing coumarins and psoralens (separation according to polarity and hydrophobicity) [30].

3.2. Method development and instrumentation

A comprehensive LC system comprises two pumps, two columns, injector, interface and detector. The interface couples the two dimensions and in the most common set-up, small volume fractions of the effluent from the first dimension are transferred *via* a multi-port switching valve into the second dimension. The methods in both dimensions should be optimized, before coupling, with respect to the sample characteristics, taking into account the parameters that affect the peak capacity [21].

For the second dimension, the time for analysis (and regeneration when a gradient is applied) is determined by the flow rate used in the first dimension and the sampling rate. The second dimension column internal diameter must be larger than the one of the column used for the primary separation (4-8 times larger [31]). Fast second dimension must be performed at high flow rates within the sampling periods, ranging from 20 seconds to several minutes, depending on the application.

Several approaches can be used to increase the analysis speed in HPLC. Monolithic columns have been extensively used in the second dimension, due to their short regeneration characteristics and high permeability [30,32-34]. Furthermore, they allow operation at high flow rates without loss in resolution. Another way to speed up the second dimension analysis is to use conventional columns packed with stationary phase of small particle size. The use of sub-2μm particle packed columns at high flow rates in the second dimension leads to increased resolution, but it requires sophisticated instrumentation, which is capable of delivering high pressures. Elevated temperatures in the second dimension have been exploited by Carr *et al.* [35], who performed ultra-fast gradients. The flow rate can be strongly accelerated without loss in efficiency, through pressure drops, caused by the reduced viscosity of the mobile phase. However, the stability of the stationary phase and analytes is of major concern, when elevated temperatures are used as a means to speed up the second dimension analysis. Fused-core materials have been used for hyper-fast comprehensive separations with high resolution, for the analysis of polyphenolic antioxidants [36].

Both isocratic and gradient programs are used in second dimension analyses. When isocratic elution is used, column conditioning, as well as the presence of negative effects due to the rapid changes of the modifier concentration on the background signals of UV or MS detectors are avoided. On the other hand, gradient elution in the second dimension is preferable, because it reduces the wrap-arounds. A wrap-around occurs when the retention time of a component in the second dimension exceeds the sampling time. This results in a broader peak, which affects the detection limit of the component.

Sometimes, several serially coupled columns can be used in the first dimension, in order to reach high efficiency. In order to diminish signal interferences and reduce band broadening , the first dimension consists usually of micro- or narrow-bore column(s) to allow focusing. The low flow rates which are used with these columns provide fraction volumes that are compatible with the conventional bore columns of 4.6 mm i.d. in the second dimension.

The interface ensures the automatic transfer of the primary column effluent to the second dimension in comprehensive LC systems. Several interface configurations have been designed and used in different applications. The *loop interface* is the most widely used interface and is based on a two-position /10-port [29,30,37], or 8-port [38-40] switching valve, equipped with two storage loops with identical volumes. In this configuration, the loops are alternately emptied and filled with primary column effluent and emptied towards the second dimension in a continuous way. In the *packed loop interface* the sampling loop can be modified by replacing the empty storage loops with loops packed with stationary phase. The mobile phase of the first dimension is preferably a weak solvent and the solutes are focused in the loop prior to their analysis in the second dimension. Fast desorption by a strong solvent should be performed by the secondary mobile phase. Greibrokk *et al*. [41], Cacciola *et al*. [42], Wilson *et al*. [43] and Venkatramani and Patel [44] have made use of the packed loop interface in their applications.

In the *stop-flow interface* approach, the columns are connected via an interface without sampling loops. One or several two-position/6-port switching valves have been used for this purpose. Köhne *et al*. have designed stop-flow interfaces using two or three 6-port valves [45,46]. Blahova *et al*. [47] have used one 6-port valve in a stop-flow set-up. Focusing of the solutes at the head of the second dimension column is necessary to avoid peak shape deterioration, therefore careful choice of the mobile phases in both dimensions is of paramount importance.

In the *interface with parallel second dimension*, the second dimension operates with two columns to carry out analyses of consecutive fractions in parallel. Columns of the same batch and system configuration (adaptation of tubing lengths) should be used when a parallel second dimension is configured. A parallel second dimension

set-up can be achieved by substituting the storage loops on a two-position/10-port switching valve by the secondary columns themselves [48].

The *vacuum evaporation interface* has been designed for the coupling of NP-LC and RP-LC by Tian *et al*. [49,50]. In this approach, the additional use of elevated temperatures, under vacuum, enabled not only the collection of the fractions eluting from the first dimension *via* the interface valve, but also accomplished the evaporation of the mobile phase solvents.

3.3. Technical problems in LC x LC

The transfer of the effluent from the first on to the second dimension must be effected in a fast and reliable way. Furthermore, the volumes of tubing, column connections and the internal parts of the valve ports should be minimal and only lead to a small contribution to the extra-column band broadening.

In comprehensive LC, the compatibility of the mobile phases in the two dimensions is also of outmost importance. The mobile phase eluting from the primary column should preferably consist of a weak solvent constituent of the second dimension mobile phase, in order to create a focusing effect (peak compression) [51]. If the solvents or solvent mixtures are not completely miscible, the combination of various separation modes becomes complicated. The coupling of NP-LC and RP-LC is highly beneficial in terms of orthogonality, but solvent immiscibilities, occurring when the first dimension apolar NP solvents mix with the aqueous second dimension mobile phases can deteriorate the separation and result in signal interferences [29,30].

Viscous fingering [52,53] occurs at the interface of two solvents with different viscosities. In a column packed with porous particles, flow instability is observed when a fluid of low viscosity displaces and penetrates a high viscosity solvent. When acetonitrile and methanol are used as mobile phases in the two dimensions of an RP-LC x RP-LC separation, viscous fingering occurs when the less viscous acetonitrile penetrates into methanol.

In comprehensive LC, the dilution that takes place at the injection of the fractions into the second dimension, by means of the interface, constitutes the primary cause of sensitivity loss.

3.4. Detection in LC x LC

All conventional LC detectors, such as photo-diode array, mass spectrometric (MS) and evaporative light scattering detectors can be used in a comprehensive 2D-LC system. Usually, a single detector is installed after the second dimension column, although an additional detector can be used to collect the first dimension data, with the first dimension separation monitored only during the optimization step.

The high speed of the second dimension analysis necessitates a very fast detector acquisition rate, otherwise loss in resolution, caused by a low number of data points, occurs. Time- of -flight MS is an excellent choice for comprehensive LC due to its high scanning rate. The 2D effluent must allow ionization of the compounds. MS detection actually adds a third dimension to the 2D system, because the mass spectrometer can identify the co-eluting non-isobaric peaks when they are not resolved by chromatography. Electrospray- and atmospheric chemical ionization are preferably used for on-line analyses, while matrix-assisted laser desorption ionization (MALDI) is usually applied to off-line collected fractions.

In comprehensive LC, a large amount of data is produced in a relatively short time. The data is visualized in 2D plots or contour plots, where the retention times in the first and second dimensions are plotted along the x- and y-axes, respectively. LC x LC currently suffers from dedicated software, necessary for both instrument control and data visualization [21].

4. Microcolumn- and nanoliquid chromatography

One of the present trends in science and technology, especially in the field of analytical chemistry, is the miniaturization of instrumentation. Great efforts have been made in order to miniaturize LC instrumentation carrying out theoretical, technological and methodological studies. Better separation efficiencies, shorter analysis times due to the lower flow rates and successive coupling with MS have thus been achieved [54]. Among these chromatographic techniques, microcolumn- and nano-LC have established themselves as complementary and/or competitive separation techniques to conventional LC [55].

The terms microbore LC, microcolumn LC and capillary LC are used interchangeably for packed microcolumns of different internal diameters (I.D.) [56]. Columns of 0.50 – 1.0 mm I.D. are usually described as micro LC, 100 – 500 µm I.D. columns as capillary LC and 10 – 100 µm I.D. columns as nanoscale LC [57].

4.1. Microcolumn LC

In 1967 Horvath and co-workers [58] introduced microcolumn LC and used 0.5-1.0 mm I.D. stainless steel columns packed with pellicular particles for the separation of ribonucleotides. Ishii *et al.* [59-61] proposed the use

of slurry-packed Teflon microcolumns, thus initiating the breakthrough in the development of microcolumn LC. The minute sample sizes and the small volumetric flow- rates improve the detection performance when concentration –sensitive detectors are used, as a result of the considerably reduced chromatographic dilution [62,63]. Nowadays, microcolumn LC is almost exclusively carried out as a research tool and in routine analysis, with slurry-packed columns of various dimensions [64,65].

Solvent selection, slurry concentration, packing pressure, surfactants used to stabilize the particle suspension, column blank material and frit selection [66,67] are the most common parameters that influence the separation efficiency of microcolumns. Small I.D. packed columns possess an almost uniform cross-sectional packing structure, therefore inhomogeneities in mobile phase flow paths are reduced [68]. More rapid trans-column diffusion between all possible flow paths and retention regions is allowed, enabling the analytes to diffuse across the entire column cross-section [69].

4.1.1.Instrumentation

4.1.1.1. Solvent delivery systems

Reciprocating pumps are the most favored solvent delivery systems in microcolumn LC, because of their rapid flow equilibrium and stability, large column back pressure compensation abilities and good possibilities for micro-gradient delivery [70-72]. Minute flows in the low µL and nL ranges, for columns with I.D.s < 500 µm, cannot be readily provided by direct pumping. For such small flows, the use of split-flow techniques is an alternative. These systems are based on the application of packed restrictor columns [73,74] or flow splitting devices based on a microflow processor concept. The latter system can be used in both isocratic and gradient mode, because it compensates for viscosity changes of the eluent, *e.g.* in gradient analysis. In gradient analysis, the microflow processor can be connected to a conventional gradient device, by means of which, part of the flow is split in a constant adjustable ratio to the microcolumn.

4.1.1.2. Injection systems

For 50-100 µm I.D. columns, injection volumes range between a few nL up to approximately 1 µL for 1.0 mm I.D. columns. Injection valves are the most favored injection systems in HPLC. Manual injections in the µL range down to approximately 20 nL can be performed with micro-injection valves equipped with a replaceable internal loop. Below 20 nL, manual valve injections can be performed by placing a split vent between the injector and the column [57]. The *moving injection technique* [75], the *static split* [76] and the *pressure pulse-driven stopped-flow injection technique* [77] can also be used for such small volumes. In all the aforementioned techniques, only a small part of the injection plug is injected on the top of the column, by controlling the injection time and flow through the injector. Automated injection in the µL range can easily be performed by commercial autosamplers, which however, require a thorough adjustment for use in the nL range. Modifications of conventional autosamplers for use in capillary LC have been described in the literature [78].

The small injected volumes or masses constitute a major problem in microcolumn LC, causing loss of detection sensitivity. This problem can be solved by the application of on-column focusing techniques [79,80], in which the sample solvent has a considerably lower eluent strength compared to the actual eluent. After arrival of the sample plug on the column top, the analytes are focused in a small plug, leading to enrichment factors of several hundreds and thus significantly increased detectability in microcolumn LC.

4.1.1.3. Tubing and connections

The tubing which is used to connect the various parts of an HPLC equipment contributes linearly to its length and to the power 4 of its radius to the extracolumn band-broadening variance. For columns of 500 µm – 1.0 mm I.D., specially designed stainless steel or polyetheretherketone or fused silica tubing of 0.25 or 0.125 mm I.D. can be employed. For smaller I.D. columns, the use of tubing is as much as possible avoided and direct connections of the column to injector and detector are preferred. Alternatively, connecting tubing with I.D.s of ≤ 50 µm is used. The length of these tubes should also be as short as possible to prevent loss in resolution [59].

4.1.1.4. Detectors

All the conventional detectors can be applied in microcolumn and capillary LC. *Refractive index (RI)* detection has in general received only small attention in microcolumn and capillary LC, because of the intrinsic difficulty arising from the fact that RI differences have to be measured in extremely small volumes. However, with the high collimating power of lasers, nL to pL volumes can easily be probed. Laser-based RI detection has, among others, been applied by Bornhop *et al.* [81] and Bruno *et al.* [82].

The *UV absorbance detector* is the most commonly used detector even in microcolumn and capillary LC with the on-column detection being the first approach

to prevent extra-column band broadening. Fiber optics have been introduced to collimate the excitation light onto the flow cell and for the collection of the UV light that has passed through the flow cell [83], however the sensitivity obtained was inferior to on-column detection. The introduction of longitudinal flow cells with an optical path length up to 3-8 mm [84] have also been tested. Photodiode array (PDA) detection has been used extensively in microcolumn LC [85-87]. Despite the small optical length of the PDA flow cell, ten times lower detection limits were reported for capillary LC, compared to conventional LC.

Fluorescence detection provides higher selectivity and sensitivity compared to UV and RI absorption. Straightforward on-column fluorescence detection is not a common approach in microcolumn LC. The majority of the applications deal with laser-induced fluorescence or detection of fluorescence emission in the packing [88,89].

Amperometric detection, potentiometric detection and conductivity are the three basic *electrochemical detection* modes. Miniaturized electrochemical detection - or the use of micro-electrodes - was first reported for open tubular LC and it was later also applied in microcolumn LC [68,69]. Amperometric detection is the most commonly used detection mode in microcolumn LC. However, only compounds that are easily oxidized and reduced at the set potential are detected, because the potential of the electrode is held at one value. By scanning the potential-or by applying triangular potential waveform to the electrode (voltammetric analysis), the number of detectable compounds that are electroactive in the potential range applied, is increased [90,91]. Voltammetric analysis has only limited applications in microcolumn LC. The main application area of electrochemical detection in microcoumn LC is bioanalysis.

Microcolumns are more suited to work with the detectors currently used in gas chromatography (GC), because they are typically operated at volumetric flow-rates of a few μL per minute. After the introduction of microcolumn LC, various types of direct introduction-based GC detectors: *flame-based* and *flameless thermionic-* , *flame photometric-* and *electron capture* detectors were used by several research groups [92-95]. Other detection principles, have also been developed for microcolumn LC, including *infrared spectrometry* [96], *chemiluminescence* [97], *inductively coupled plasma atomic emission spectrometry* [98] or *evaporative light scattering* [99]. These detection techniques have been applied with limited success in microcolumn LC and definitely not for routine use, mainly because they are either too selective or not robust enough.

4.1.2. Hyphenation

Microcolumns are suitable for coupling with secondary separation techniques, resulting in a multidimensional chromatography scheme. Thus, microcolumn LC has been successfully interfaced with thin-layer chromatography using infrared detection [100], conventional LC [101], microcolumn LC [102], gas chromatography (GC) [103-106], supercritical fluid chromatography (SFC) [107] and capillary electrophoresis (CE) [108].

In microcolumn LC - GC a liquid mobile phase is converted into a GC-compatible, *i.e.* gaseous- sample. Several interfaces have been designed for this purpose such as on-column injectors [103] and loop-type interfaces [104], pyrolysis interfaces and multi-capillary stream splitters. Microcolumn LC – GC hyphenation belongs to the heart-cutting techniques, which means that only a part of the first dimension effluent is introduced onto the secondary separation system, as already has been described in section 3.1. High sampling frequencies have been achieved by coupling microcolumn LC to capillary electrophoresis. Lemmo and Jorgensson [108] coupled microcolumn size exclusion chromatography (SEC) and CE to study protein standards, obtaining high efficiencies in short period of time.

Microcolumns are also well suited for coupling with MS and can be either directly coupled to the ion source or *via* transfer lines. The latter increases band broadening adding to the overall band broadening caused by MS itself. The breakthrough in microcolumn LC-MS interface came with the coaxial interface of a microcolumn with continuous flow-fast atom bombardment (CF-FAB) ionization source [109]. The number of applications developed with microcolumn LC-CF-FAB is extensive. The electrospray (ES) ionization was almost simultaneously introduced with CF-FAB and has become a very popular technique for analysis of biomolecules. The coupling of an ESI interface to microcolumn LC is relatively easy [110]. Furthermore, the LC-ESI-MS interfaces exhibit an almost linear relationship upon the analyte concentration. Other types of interfaces also have been used for coupling micro- and capillary- LC with MS, such as electron impact and chemical ionization [111] and the particle beam interface [112].

4.2. Nanoliquid chromatography

Nano-LC was first introduced by Karlsson and Novothy in 1988 [55] and since then it has been used as a complementary and/or competitive separation method to conventional HPLC, offering higher efficiency, shorter analysis time and better compatibility with MS, due to the relatively low flow-rates (40-600 nL min^{-1}) that allow the transfer of the entire effluent from the column into

the MS [113]. Nano-LC is an environmentally safe and cost-effective technique. It employs minute volumes of mobile phases, producing minimum waste and also uses small amounts of stationary phases, which is beneficial when expensive packing materials are used. Nowadays, nano-LC, as a miniaturized version of HPLC brings significant improvements in analytical chemistry methods. It has been applied to the analysis of numerous compounds in different areas, such as pharmaceutical, environmental and proteomic, with the latter being the major application field, mainly because of the very low sample requirements. Nano-LC has been especially applied in protein sequencing, peptide mapping or determination of post-translational modification of proteins. An interesting review on applications of nano-LC in the above fields has been reported by Hernandez-Borges *et al.* [54].

4.2.1. Capillary columns

Capillary columns of 10 -100 μm I.D., used in nano-LC, contain either silica-modified particles of 3 -5 μm, monolithic supports [114], or wall coated with appropriate materials [115,116]. In nano-LC the use of pre-columns is recommended, as in conventional HPLC, since capillaries can be easily blocked at the inlet, when real samples are injected. Pre-columns can also be used for sample clean-up and pre-concentration, on-column focusing *etc.* [117].

In the case of packed capillaries, because of the high cost of the commercially available columns and due to the limited types of stationary phases available, many research groups manufacture their own capillaries. Reverse phases C2, C8 and C18 have been used in nano-LC for proteomic and/or pharmaceutical analysis [114], while silica particles modified with chiral selectors like glycopeptides antibiotics, cyclodextrins *etc.* have been applied to enantiomeric separations.

Besides packed capillaries, monolithic materials have also been used in nano-LC for application in different preparation processes. A large variety of monolithic nano-LC columns are commercially available, although many of these columns are laboratory-made by *in-situ* polymerization. Four types of monolithic capillary columns can be found nowadays: particle-fixed, silica based, polymer based and molecular imprinted monoliths [118].

The solvents most commonly used in nano-LC mobile phases are the same as those employed in conventional HPLC, with reversed-phase mode being the most popular, while the normal-phase mode has not been so frequently used. The use of capillary columns in nano-LC is well suited for gradient temperature programs. The small capillary diameter allows a fast heating or cooling of both stationary and mobile phases [119].

4.2.2. Sensitivity in nano – LC

Due to the small sample volumes injected (20 - 60 mL), nano-LC cannot be considered as a technique with high sensitivity. In order to overcome this problem, several studies have been conducted to obtain improved sensitivity. Thus, the use of appropriate detector systems (MS, UV detection with a large path length), on-column focusing and 2D separation resulted in enhanced sensitivity. In the on-column focusing approach [120], a 75-μm I.D. column was used for the nano-LC-MS trace level determination of organophosphorous pesticides. The authors developed a 500-nL injection allowing a 100-fold decrease in the LODs by using water in both, the sample and the initial mobile phase composition. The 2D separation approach was successfully applied to the nano-LC analysis of peptides [121]. The sample was focused on a column with a larger I.D. allowing the use of higher flow-rates, *e.g.* μL min^{-1} and the transfer of analytes in the analytical column.

4.2.3. Generation of flow in nano-LC

The split-flow technique is the most widely used method for generation of nanoflows for LC systems employing capillary columns with I.D.s ≤100 μm. A T- union with zero dead volume is connected to the micropump via the split-flow device. The other two ends are connected to an empty capillary (waste) and to the packed capillary. The split ratio can be controlled by modifying the length and I.D. of the empty capillary. With this arrangement it is easy to obtain nanoflows in the isocratic mode. Reproducible gradient elution is however difficult to achieve with home-made devices, because the different viscosities of the mixed solvents cause backpressure fluctuations. Furthermore, the change in the mobile phase is transferred to the column with a small delay [54].

In another type of pump, a direct flow is produced as follows: the solvents are mixed in an appropriate chamber and the gradient profile is controlled by the volume of the chamber, the applied gas pressure and the flow-rate [122]. Capiello *et al.* [123] used a conventional HPLC pump for flow-gradient generation. The device comprised an electric actuator, computer-controlled multiposition HPLC valve, equipped with 6 loops, each one containing a selected mixture of solvents of different strengths. Murata *et al.* [124] developed a gradient generator, to be used in the analysis of peptides by nano-LC-MS, employing a split tubing array (STAR) and a mixing chamber with a relatively large volume.

Takeuchi *et al.* [125] proposed a simple method to produce stepwise gradient elution in micro-LC. The two solvents were positioned in two loops connected to different switching valves. In all these systems it is easy to change the gradient composition, but both pressure and flow-rate are slightly perturbed during valve switching.

Apart from the laboratory-made devices, instrumentation for nano-flow generation with or without split-flow systems is nowadays commercially available. The delivery of the solvents into the mixing chamber and the flow are automatically controlled taking into account the changes of the backpressure.

4.2.4. Detectors

UV detectors are the most commonly used in nano-LC as in conventional HPLC, because of their simplicity, low cost, broad range of applicability and the availability for on-column detection. However, due to the short path-lengths of the capillaries, sensitivity is limited when on-column detection is applied. For this purpose, the use of Z-cells [126,127] as well as on-line or off-line preconcentration procedures have been applied to enhance sensitivity [128]. Fluorescence detection has only been used in a few nano-LC applications [129-131], although it provides higher sensitivity and selectivity than UV-Vis detection, also because of the short optical path-lengths of the capillaries. Electrochemical detection has also not found widespread application in nano-LC [129-131], despite the increased sensitivity it offers. This could be attributed to the fact that this type of detection is suitable for electrochemically active compounds only. MS detection is becoming more and more popular for those working with miniaturized techniques, because MS can be easily coupled with nano-LC through the several nanospray interfaces developed. Nano-LC-MS coupling is highly compatible because of the low flow-rates (50-600 nL min^{-1}) used. A number of the nanospray devices developed are commercially available, although most of them are laboratory made. In most cases, the capillary column is connected to the emitter tip through a zero dead volume union attached to a power supply. Further details can be easily found in relevant literature [113,132,133].

5. Chip-based liquid chromatography

The demand of methods combining the possibility of analyzing minute samples in the shortest time possible and integrating in the very same instrument both sample pretreatment and separation has led to the development of micro-fabricated devices (named µ-total analysis system, µ-TAS). The main advantages of these devices are the low LODs, the increased peak capacity and sample loading and the minimization of the number of handling and transfer steps they offer [54]. Many of these approaches, using centimeter-size glass, silicon or plastic chips have been successfully applied to proteomic analysis [134,135].

Although the first µ-TAS chip was actually designed for pressure driven LC [136], most of the on-chip separations developed up to now have been focused on electrokinetically driven separation techniques such as Capillary Electrophoresis (CE), Capillary Electrochromatography (CEC) and Micellar Electrokinetic Chromatography (MECK). A number of problems that have hindered the development of HPLC chips are among others the following: the difficulty of integrating on-chip injectors and mechanical valves, the lack of easy flow control, the on-chip application of high pressures and the manufacturing of columns with sufficient sample capacity [137]. However, the situation with chip-based LC has been changing and several approaches have been developed which show that the technological problems, at least partly, have been solved. For instance, Yin *et al.* [135] developed a system that is now commercially available while Agilent and Xie *et al.* [138] reported a similar device. Both systems make use of columns packed with commercially available beads. The pressure is applied by an external pump in Yin's device and by an integrated pump based on electrolysis in Xie's system. Ericson *et al.* [139] used a monolithic column on-chip and an external pump. Although these systems offer the advantages related to a chip-based format, *i.e.*, low dead-volume on-chip connections, suitability for small volume samples and ease of automation, they still miss an important piece of progress that can be made by micromachining, namely an entirely micromachined separation column.

Micromachining is needed to optimize column performance. Most columns operate at plate heights around h_{min} (where h, plate height) [140]. Much lower values of h_{min} could be obtained if the stationary phase were made entirely ordered, for instance by micromachining the entire column. The open tubular column, which would be the ultimate ordered column, unfortunately does not offer sufficient sample capacity to be practically useful [141].

Several technological challenges remain before micromachined columns can become standard components in chip-based LC systems. Thus, partly porous columns must be manufactured in order to ensure sufficient sample capacity. It is also very important to have a uniform coating of the stationary phase, because non-uniformities in the coating will almost have the

same dentrimental effect on the plate height as non-uniformities in the flow-path. One way of homogenizing the flow-path, further than allowed by particle packing, is by using organic or inorganic monoliths as the stationary phase. Monolith development for chip-based LC is therefore an active research area [142]. Gzil and co-workers [143] showed however that even in the best polymerized monolithic columns, the lack of structural order will still limit their separation performance.

Another technological challenge is the application of high pressures to the chip, a problem which is almost solved, taking into account the interfacing with external pumps. A further hinderance is the necessity to inject a narrow plug of analyte onto the column, in order to limit the contribution of the injection to plate height. Several interesting approaches have been reported, as for instance, that of Reichmuth *et al.* [142], employing a hydrophobic valve and those of the off-line injection valves and the on-column injection channel or slit as chosen by Yin *et al.* [138] and Eghbali *et al.* [144].

5.1. Recent advances in the use of monoliths in micro- fluidic devices

Micro-fluidic devices have been applied in almost all scientific areas of analytical and diagnostic importance, over the past decade. Environmental and clinical analysis, proteomics and genetics are only some of the hot topics in which micro-fluidic formats have found widespread acceptance [145-149]. Micro-fluidic systems offer the possibility of integrating different analytical operations, *e.g.* sample pretreatment and separation, in one single chip. Monolithic materials been extensively exploited in micro-fluidic devices to modify, control, manipulate and improve the speed and efficiency of an analytical process. Despite the numerous publications dedicated to micro-fluidic formats, it was only as recently as 2000, that Ericson and his co-workers [139] reported on the first micro-fluidic chip integrating a monolithic phase, as the separation column. In this work, an acrylamide-based monolithic stationary phase was prepared *in-situ* in the channels of a quartz micro-fluidic chip.

A number of monolithic materials are nowadays available, exhibiting different pore sizes, porosities, and a large variety of surface functionalities. Monolithic materials can be easily prepared in micro-fluidic channels by *in-situ* polymerization. Porous polymer monoliths, silica-based monoliths and related homogeneous porous polymer gels have been applied in the preparation of ion-permeable membranes, preconcentrators, extractors, separation columns, electrospray emitters, micro-valves, electrokinetic pumps, micro-reactors and micro-mixers in micro-fluidic devices. Due to their unique properties, monoliths have almost entirely replaced particle-based materials for many applications within micro-fluidic devices. Preparation of monolithic materials for use in micro-fluidic systems can be easily carried out by photo-initiated polymerization employing customized photo-masks [150]. Functionalization of the whole monolithic bed can be performed afterwards, by applying photo-grafting techniques [151]. The preparation of particulate columns in micro-channels is much more cumbersome, as beads need to be contained by frits previously fabricated in the channels and then cautiously compacted in order to avoid voids in the packed column. Micromachined perfectly ordered pillar array columns can however efficiently replace the use of packed beds for microchip-HPLC separations [144].

The progress in micro-fluidic chip fabrication technology, including the development of protocols for bonding plastic substrates, has led to the increasing use of plastic devices [146,147]. Over the last 10 years, monolithic columns have been used more extensively in electrophoretically driven methods than in chip-based micro-LC, mainly due to the inability of most micro-fluidic formats to withstand the high back pressures required. However, new advances in the micro-fabrication and bonding techniques used in the production of micro-fluidic devices, as well as improvements in the connection of the chips with macro-components, like injectors, pumps and detectors have led to significant progress in this area.

Monolithic materials within micro-fluidic devices have also been used for the micro solid phase extraction (μSPE), clean-up and preconcentration of analytes [152-154]. Within each of these analytical procedures, the application of monolithic materials has involved one or more of the following approaches: (a) use of silica monoliths or highly hydrophobic organic polymer monoliths, (b) fabrication of monolithic frits for silica bead entrapment, (c) preparation of monolithic matrices with embedded silica beads and (d) preparation of affinity monoliths.

5.1.1. Electrospray emitters

The number of publications dealing with the coupling of micro-fluidic chips with ESI-MS has been growing steadily in the last decade. However, only a few reports have explored the exploitation of monolithic materials in the fabrication of on-chip electrospray emitters. Oleshuck and his co-workers have developed a number of monolith-based electrospray emitters prepared in capillaries [155] and glass chips [156]. They also investigated different polymeric materials, *i.e.*, polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA) and cyclic olefin copolymer (COC) for the fabrication of cheap and disposable chips integrating hydrophobic polymer

monoliths as electrospray emitters [157]. Due to the superior properties of COC substrates, COC chips showed the highest chip-to-chip reproducibility.

5.1.2. Micro-valves

One-way valves based on hydroxyethyl methacrylate (HEMA)-ethylene glycol dimethacrylate (EGDMA) have been prepared *in situ* in polycarbonate chips by applying a double UV-exposing method [158]. The valve is closed when the hydrogel is swollen and is opened when the force generated by the flow pressure becomes greater than the elastic force of the hydrogel, causing the head of the HEMA valve to retract. More recent advances in this area include the development of thermally actuated valving systems, based on hydrogels which are prepared from N-isopropylacrylamide (NIPAAMm) cross-linked with N,N´-methylenebis acrylamide (MBAA). Wang *et al.* manufactured micro-valves based on NIPAAMm gels, prepared off-chip by adding N,N,N´,N´-tetramethylethylenediamine (TEMED), which acts as an accelerator, and ammonium persulfate (APS), which acts as an initiator, to the polymerization mixture [159]. Valve closing and opening times were relatively rapid, at 4.5 and 5-12 s, respectively. Other micro-valves based on the use of nano-composite materials formed by entrapment of nano-particles (gold colloids and gold shells) with different optical properties into thermally responsive NIPAAm gels, were reported by Sershen *et al*. [160]. Two different valves, based on the two distinct nano-composites were then fabricated in the channels of a micro-fluidic chip. In another study, Chen *et al.* [161], reported the use of light-actuated micro-valves based on NIPAAm gels in COC micro-fluidic chips. The valves were actuated by conversion of the focused light, emitted from a quartz halogen lamp, into heat, which resulted into swelling of the polymer due to its thermo-responsive properties. The valve opening and closing response times decrease with increasing concentration of THF in the polymerization mixture.

5.1.3. Electrokinetic pumps

Electroosmotic pumps (EOPs) is a challenging alternative of other types of valves, which can be used within micro-fluidic formats. Monoliths have been applied in the fabrication of EOPs within many forms of micro-fluidic chip. EOPs' function is based on the generation of an electroosmotic flow upon application of an electric field. EOPs present several advantages compared to other types of pumps used in micro-fluidic platforms, such as the rather easy and low-cost fabrication and the generation of constant and pulse-free flows. Chen and his co-workers [162], presented silica monolithic columns fabricated within fused silica capillaries and showed that they can serve as high-pressure EOPs, due to the micrometer size through- pores with a large negative surface charge density, which produces large hydrodynamic resistance. Silica-based monoliths are superior to polymeric monoliths in their mechanical strength and high stability in both aqueous and organic solution. Flow-rates of 200 nL to 2.5 μL min^{-1} were obtained with 6-cm long columns (100μm I.D.) by varying the voltage from 1 to 6 KV. Nie *et al.* [163] fabricated on-chip EOPs based on monolithic silica capillary columns. The columns were inserted into the micro-channels of a PMMA chip fabricated by direct micro-milling. Bubble-free flow-rates up to 0.6 μL min^{-1} were obtained at 2 KV with EOPs based on triple silica columns.

6. Ultra performance Liquid Chromatography (UPLC)

Ultra Performance Liquid Chromatography (UPLC) can be considered to be a relatively new direction of liquid chromatography. Throughout the history of HPLC there has been a trend to use smaller particles as packing material. It is well known that as particle size decreases to less than 2.5 μm, there is a significant gain in efficiency, and thus resolution, and the efficiency does not diminish at increased linear velocities or flow rates according to the Van Deemter equation [164]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance.

UPLC takes advantage of the huge progress achieved in particle chemistry performance, system optimization, detector design and data processing and control. The use of sub-2 μm particles and mobile phases at high linear velocities and instrumentation that operates at higher pressures than those used in HPLC, resulted in dramatic increases in resolution, sensitivity and speed of analysis. This new category of analytical separation science retains the practicality and principles of HPLC while creating a step function improvement in chromatographic performance [165,166].

In conventional HPLC the use of continuously smaller sized packing material particles leads to higher column back-pressure. Small column diameters like 2.1 or 1.0 mm could also cause similar problems and disable their use under the conventional conditions. In order to overcome the pressure limitation of conventional equipment, shorter columns packed with small particle diameter particles were used. However, the use of UPLC on a routine basis in the laboratory, required further improvement in some practical aspects, such as sample introduction, reproducibility and detection. Ultra high

pressure columns required extremely narrow sample plugs to minimize any sample volume contribution to peak broadening. The advent of UPLC demanded also the development of a new instrumental system for liquid chromatography, which would take advantage of the separation performance (by reducing dead volumes) and withstand the pressures (about 8,000 to 15,000 psi, compared with 2,500 to 5,000 psi in HPLC). Efficiency is proportional to column length and inversely proportional to the particle size. Therefore, the column can be shortened by the same factor as the particle size without loss of resolution [167].

6.1. UPLC system adjustments

6.1.1. Small particle chemistry

UPLC must use novel porous particles that can withstand high pressures, in order to maintain retention and capacity similar to HPLC. Silica based particles have good mechanical strength, but they work through only a limited pH range and present tailing of basic analytes. On the other hand, polymeric columns can overcome pH limitations, but suffer from low efficiencies and limited capacities. In 2000, a first generation hybrid chemistry that took advantage of the best of both the silica and polymeric column materials was presented [168]. These columns (X-Terra) were produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups and are mechanically strong, with high efficiency and can operate over an extended pH range. However, the kind of enhanced mechanical stability required for UPLC dictated the development of a second generation bridged ethane hybrid (BEH) technology [169]. These 1.7 µm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix.

Packing reproducible and rugged columns with 1.7 µm particles was also a challenge that needed to be overcome. Smoother interior surface of the column hardware, re-designing the end frits to retain the small particles to resist clogging and packed bed uniformity are required. The latter is critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations. Further, at high pressures and with column diameters typically used in HPLC (3.0 to 4.6 mm), frictional heating of the mobile phase can cause a non uniform flow, resulting in loss of performance [170]. To minimize the effects of frictional heating, smaller diameter columns (1–2.1 mm) are typically used for UPLC [171,172].

6.1.2. Pumps

A considerably greater pressure range than that achievable by today's HPLC instrumentation was required to take full advantage of UPLC's superior peak capacity. Working with a 15 cm long column packed with 1.7 µm particles, at the optimum flow rate for maximum efficiency, results in a pressure drop of about 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures, in both the gradient and isocratic separation modes, and that can compensate for solvent compressibility, was required [165-167].

6.1.3. Sample injection

Sample introduction is also critical in UPLC. Injection valves, either automated or manual, utilized in conventional HPLC instrumentation, are not designed and hardened to work at extreme pressure. To protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. The sweep volume of the injection device should also be minimal to reduce potential band spreading. A fast injection cycle time is required to exploit the speed afforded by UPLC which in turn, requires a high sample capacity. Low volume injections with minimal carryover are also needed to take full advantage of the increased sensitivity benefits [165-167].

6.1.4. Detectors

Half-height peak widths of less than one second are obtained, with 1.7 µm particle-packed columns, posing significant challenges for the detector. For an accurate and reproducible integration of an analyte peak, the detector sampling rate must be adequately high to capture enough data points across the peak. Further, the detector cell must have minimal dispersion (volume) to maintain separation efficiency. The sensitivity increase for UPLC detection should be 2–3 times higher than with HPLC separations, depending on the detection technique that is used. Conventional absorbance based optical detectors are concentration sensitive detectors. For UPLC use, the flow cell in standard UV/Visible detectors would have to be reduced in volume-size to maintain concentration and signal, while avoiding Beer's law limitations. MS detection is significantly enhanced by UPLC. Increased peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) promotes increased source ionization efficiencies (reduced ion suppression) for improved sensitivity [165-167].

6.2. Advances in UPLC instrumentation

Early work dealing with the development of ultra high pressure reverse phase liquid chromatography methods were those of MacNair *et al.* [171,173] who tested a UPLC system in connection with packed capillary

columns using 1.0 or 1.5 µm nonporous ODS-modified particles. They also invented a static-split injection technique, which was necessary to achieve high column efficiencies and withstand high pressures. Working pressures (496.8 MPa, 72,000 psi, respectively) used at their experiments was referred to be the highest pressure used in LC. The problems of their ultra high pressure experiments were possible thermal effects and pressure dependent retention effects. Another work by Wu *et al*. [174] tested ultra high pressure capillary liquid chromatography using fused silica capillaries packed with nonporous 1.5 µm isohexylsilane-modified (C6) particles, stating that only capillary columns should be used in UPLC so as to facilitate frictional dissipation. Experimental pressure-balance injection valve was used for sample introduction and the comparison with previously described static-split injection was made. Upper pressure limits allowed using maximum 100 MPa [175].

Standard HPLC technology (pumps, injectors, and detectors) simply did not have the required capability to take full advantage of sub-2 µm particles. However, many of the early UPLC systems needed in-house modification of commercial products at the laboratory itself and also the own manufacturing of analytical columns often capillary columns, as was stated above. To overcome these problems, in early 2004, the first commercially available UPLC system that embodied these requirements was described for the separation of various pharmaceutical related small organic molecules, proteins, and peptides; it is called the ACQUITY UPLC™ System [176].

The Acquity UPLC system adjustments involve (a) a binary solvent manager using two individual serial flow pumps with a 15,000 psi pressure limit, to deliver a parallel binary gradient mixed under high pressure, along with in-built solvent degassing and solvent select valves, (b) a sample manager (including the column oven), (c) a detector and (d) an optional sample organizer. Low dispersion is maintained through the injection process employing pressure assist sample introduction. Sample injection is characterized by fast injection cycles (25 s without a wash and 60 s with a dual wash), low injection volumes, negligible carryover and temperature control (in a range 4–40ºC), which together contributes to the speed and sensitivity of UPLC analysis. Using the optional sample organizer, the sample manager can inject from samples from up to 22 microtiter plates. The sample manager also controls the column heater. The detector uses fiber optic flow cell with 10 mm path-length and 500 nL cell volume, offering high sampling rate, minimal dispersion and high acquisition rate (20–40 points s^{-1}). System volumes are minimized so as to maintain sensitivity, speed and resolution of analysis. The UPLC system is connected with specially designed Acquity UPLC columns containing X-Terra sorbent of second generation. This hybrid material has a particle size of only 1.7 µm and uses bridged ethylsiloxane / silica hybrid (BEH) structure. BEH technology ensures the column stability through a wider pH range (1–12) and under high pressures, comparing to generation one X-Terra sorbent or conventional stationary phases. Acquity UPLC columns are available with C18, Shield RP-18, C8 and Phenyl stationary phases [166,175].

When transferring methods from HPLC to UPLC, the AQUITY UPLC calculator can be used for a quick, automatic transfer and optimization. There are two simple steps to be followed for a successful method migration: (a) column particle size and length adjustment to keep the L/dp constant and (b) flow-rate correct scaling for new column geometry, particle size and separation duration.

7. Concluding remarks

At a time when many scientists have reached separation barriers with conventional HPLC, UPLC seems to be the possibility to extend and expand the utility of chromatography. In conclusion, the main advantages of this relatively new approach of chromatography are: increased sensitivity, faster analysis through the use of a novel separation material of very fine particle size, reduced operation cost, less solvent consumption and increased sample throughput. However, a negative aspect of UPLC could be the higher backpressure than in conventional HPLC. Due to increased pressure, UPLC requires more maintenance and reduces the life of the columns of this type. This backpressure can be reduced by increasing the column temperature. Overall, it seems that UPLC can offer significant improvements.

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