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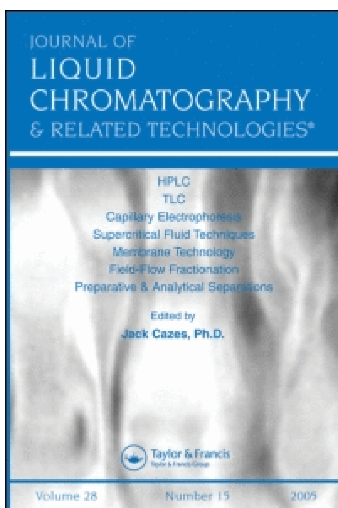

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High Speed Liquid Chromatography for In-Process Control

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Abstract: High speed liquid chromatography (HSLC) represents a significant advance on HPLC theme because improves the in-process control methods thanks to faster analyses, as well as reduces operational costs and increases sample throughput. Therefore, HSLC let us control manufacturing processes more efficiently and increase industrial productivity. The most straightforward approaches to fast separations are the short columns packed with microparticles and monolithic columns in combination with high flow rates. Using current HPLC technology, HSLC can be successfully implemented for routine work.

Keywords: High speed liquid chromatography, Process control

INTRODUCTION

HPLC is a powerful analytical technique that is now included in the restrictive group of total analysis systems (TAS). This group includes automated and computer controlled techniques able to separate, identify and determine all the components of a complex sample, including organics, ions, synthetic polymers, and biomolecules with precision, sensitivity and reliability. Consequently, HPLC satisfies most of the requirements demanded by modern industry of analytical techniques. For these reasons, HPLC is an indispensable tool in the laboratories, so different as pharmaceutical industry, agrochemical analysis, food technology, environmental analysis, forensic studies, etc.

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Nevertheless, when the number of analysis to be carried out by a laboratory is very high, when real-time process control is mandatory, or simply when a laboratory wants to increase its productivity, classic liquid chromatography may be slow. As the classic liquid chromatography is unable to solve a complex mixture of substances in seconds or as maximum in some few minutes, high speed liquid chromatography–also named fast HPLC–emerges like a solution of agreement between resolution and analysis time. The concept of performing HSLC is not new and some authors attribute it to Martin and Synge,[1,2] who postulated that efficiency increased as the particle size decreased. This is as a result of the relatively small intra-particulate mass transfer resistance due to the short diffusion distances and the small contribution of Eddy diffusion to the plate height.[3] Since then, different authors, among them are Halasz, Guiochon, Knox, Scott, Dong, Horváth, Kirkland, Unger and Erni, have paid special attention to the theoretical bases of HSLC with the purpose of facilitating the instrumental advances, as well as the development of new chromatographic methods more faster and reliable. In this sense, column technology (new packing material design, new bonded-phases, and column construction and format) has been mainly responsible for the advances in this field.

In its conventional format, HSLC makes use of columns of standard diameter (4.6 mm) with length up to 10 cm, generally with stationary phases of 3 μm particle size and working at a flow rate of mobile phase higher than 2 mL min^{-1}. As it has been demonstrated,[4–6] the use of small particles in short columns allows an increase in the flow rate without marked losses in effectiveness, as these particles are less resistant to mass transfer. Under these conditions, an analysis can be performed in seconds or few minutes. In our opinion, the diameter of these columns should be lower than 3 mm, because such columns allow to reduce flow rate up to five-fold, approximately, which reduces the cost of the analyses and environmental pollution. Related microbore columns increase effectiveness, sensibility and precision of the chromatographic method. In order to improve resolution, modern stationary phases of fine granulometry (2–1.5 μm, porous or non-porous) could be used although their current price limits their applications.

Another alternative may be the use of monolithic stationary phases, characterized by high porosity and permeability. The analysis time reduction is achieved by increasing flow rate up to 9 times the usual one in a conventional column although the high consumption solvent and the price of the columns are important drawbacks. Furthermore, these monolithic columns may generate on basic compounds more peak asymmetry than good classical full-endcapped columns.[7]

In a few words, it should be realized that the limits of speed in HPLC can be discussed from the following viewpoints: chromatographic theory (the roles of column design, packing materials, temperature), instrumentation (requirements for HPLC instrument components), and practical needs (requirements of typical users in research, method development and quality control).[8]

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Considering the practical needs, the reasons for using HSLC are the new fields of applications where fast analyses are essential, such as fermentation control, investigation of fast kinetics, pharmacokinetic studies, and process control in general. Besides, for other applications where speed is not as important as the aforementioned ones, HSLC can also be used advantageously with the aim of getting faster results, more analyses per unit time, more analyses per instrument and reduced cost per analysis. An additional benefit of HSLC is the ability to optimize an HPLC separation in a faster time. Finally, decreased solvent consumption is becoming increasingly important, to minimize costs and waste disposal problems.

On the other hand, in today's competitive environment, high-throughput analytical techniques are essential to both the optimization of development time cycle as well as real-time decision-making. Various analytical techniques have been used to support process development, optimization of the synthetic route, and quality control. The majority of these analyses have been performed by HPLC because of the detailed information obtained.[9] Therefore, it is logical that high speed HPLC separations can have a significant effect on industrial productivity.

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COLUMN TECHNOLOGY

Packed Short Columns

At the beginning, HSLC has been practiced on short columns about 50 mm in length and 4.6 mm inner diameter packed with 3–5 μm particle size. When the required chromatographic effectiveness is not very high, a microbore (1–2.1 mm i.d.) short column (30–50 mm length) with conventional 3 μm stationary phases is the choice, due to the fact that column and operation price are cheap and the compatible instrumental set-up is also relatively simple and economical.

It should be kept in mind that critical in this respect is the ratio of the extracolumn volume to the column volume itself. The smaller the column used, the stronger the influence of the extracolumn volume of the instrument. Therefore, in order to maintain the true efficiency of short columns, some attention must be paid to special requirements of high speed instruments as regards the injection valve, the detector and the connecting tubes (see Instrumental requirements) with the purpose of reducing the instrumental bandwidth, also known as extracolumn band broadening. These detrimental extracolumn effects must thus be minimized, especially for microbore short columns employed for fast separations (fast gradients and early eluting peaks) because of smaller retention volumes of analytes.

Now, short columns packed with porous and non-porous 1–2 μm particle size, are offered by different manufacturers for fast and efficient separations. In fact, it is the logical approach to improve resolution although the

commercial columns are still expensive compared to standard HPLC columns (for novel concepts in the design of silica packing, see the reviews of Unger et al.[10] and Kirkland[11] and their references). In addition, some columns packed with porous particles do not support backpressures higher than 120 bar and, for this reason, the flow rate is limited and consequently, the speed of the separation is limited, too. The main advantage of non-porous packing is the rapid mass transfer between the mobile and stationary phases due to the short diffusion distance in the thin retentive layer of the particles. However, these columns possess low specific surface area and low dead volume then, the column loadability is also reduced. Besides, the column pressure drop is high even for normal flow rate at ambient temperature. Obviously, this problem increases quickly when particle size is smaller because pressure is inversely proportional to the square of particle diameter. This drawback can be mitigated working at high temperature in order to decreasing mobile phase viscosity. Nevertheless, not many efficient stationary phases are currently available to withstand high temperatures. Another concern is the possible degradation of labile analytes at elevated temperatures ($>80^{\circ}$C), which may ultimately limit the wide use of high-temperature HPLC technique, unless the analysis time cycle can be reduced to the point at which it favorably competes with thermal degradation kinetics.

Another way to overcome this obstacle consists on using pumps able to operate at much higher pressures than standard HPLC delivery systems, but these equipments are more expensive than the standard ones. This technique has been named ultrahigh–pressure high performance liquid chromatography (UPHPLC).[12]

In summary, the speed issue is being approached from several directions: smaller particles ($<5\ \mu$m), monolith columns, higher pressures (>600 bar) and higher temperatures (as high as 150°C). Among them, the most straightforward approaches to high speed separations are the short columns packed with small particles and monolithic columns in combination with high flow rates, which monopolize most of the applications because they are the most popular options in HSLC.

Monolithic Columns

In Guiochon's opinion,[13] the invention of monolithic columns is a major technological change in column technology in the last century. Polymeric monolithic stationary phases offer an alternative to the classical microparticulate sorbents, bringing important advantages to sample analysis. In contrast to the traditional stationary phases that consist of packed particles, the monolithic separation medium is made of a continuous, rigid polymeric rod with a porous structure. The lack of intraparticular void volume improves mass transfer and separation efficiency. Its greater porosity and hence greater permeability allows to operate at higher flow

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rate and, in this way, fast chromatographic separations can be achieved at low backpressures, resulting in a five-fold increase in sample throughput. Together with this, the easiness of stationary phase preparation, make monolithic columns more interesting for some application versus common columns packed with beads.

Different shapes of monolithic stationary phases are currently commercialized for HPLC separations. The macroporous disc format was originally designed for fast proteins separation,[14] and now are prepared with different modified polymeric substances (polystyrene, polymethacrylate, modified silica or modified cellulose) with the purpose of obtaining the desired separation mechanism (ion exchange, reversed phase, hydrophobic interaction or bioaffinity). The monolithic stationary phases made from organic polymers (reversed phase, ion-exchange) are polymerized inside the chromatographic column under controlled conditions. On the contrary, silica-based monolithic columns (reversed phase) are first prepared in moulds and later encased within peek tubes.[15] Although it has been reported that the monolithic silica columns are only useable at pH levels 2–10, the large through pores and high surface area are well suited for both small molecules and large biopolymers.

In the last few years, some authors have studied the behavior of monolithic columns in relation to conventional and packed high speed columns, with the purpose of examining its capacity as an alternative stationary phase in HSLC.

Cabrera et al.[16] describe a monolithic type of HPLC column, the SilicaROD column, which permits the fast HPLC separation of compound mixtures in industry, especially for the quality control of products, within a few minutes. A comparison of the separation of three parabenes on the SilicaROD RP-18 column (83 × 7.2 mm i.d.) and on a small HPLC column (Micra, 33 × 4.6 mm i.d., 1.5 μm NPS) is provided. The analysis time is the same as both columns (1 min). However, the resulting backpressure of the ROD column is less than half of the backpressure of the Micra column (100 vs. >200 bar). On the other hand, a unique feature of SilicaROD columns for the optimization of HPLC separations with respect to time is to successfully apply high flow rates combined with flow gradients. So, the active ingredients of the medicine Ilvico, namely paracetamol, caffeine and bromopheniramine were separated within 2.5 min using a SilicaROD RP-18 end-capped (93 × 4.6 mm i.d.) and acetonitrile–0.02 M phosphoric acid (10/90; v/v) as mobile phase in flow gradient.

Smith and McNair[17] have also studied the behavior of silica-based monolithic ODS columns by means of a number of parameters, such as the backpressure, selectivity, resolution, re-equilibration time, run-to-run and column-to-column precision. The analytical columns employed were: Chromolith SpeedROD RP-18e (50 × 4.6 mm i.d.), Phenomenex Luna (50 × 4.6 mm i.d., 5 μm), Waters Symmetry (50 × 4.6 mm i.d., 3.5 μm), Waters XTerra (50 × 3.0 mm i.d., 3 μm), Optimize Velocity (50 × 4.6 mm

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i.d., 3 μm), Varian Chrompack (50 × 4.6 mm i.d., 3 μm). The seven-component test mixture (benzamide, *N*-methlbenzamide, benzyl alcohol, acetophenone, ethylparaben, propylparaben and biphenyl) was used to assess the selectivity of five particulate columns and the Chromolith SpeedROD. In all cases, a good agreement was found regarding the behavior between particulate columns and monolithic ones. In addition, these last ones offer important advantages: lower backpressures, higher flow rates without significantly compromising column effectiveness, shorter re-equilibration times (suitable for gradient mode) and a reduced solvent consumption thanks to shorter analysis times.

Two monolithic columns (Chromolith Performance RP-18e, 100 × 4.6 mm i.d. and SpeedROD RP-18e, 50 × 4.6 mm i.d.) have been investigated, by Wu et al.[18] as a possible tool for reducing separation time in reversed-phase HPLC without significantly sacrificing effectiveness or resolution for drug process development. The pressure drop, retention, efficiency, selectivity, and tailing factors of monolithic columns were compared to those of conventional 3 μm and 5 μm particle packed columns (YMC C_{18}, 5 μm, 120 Å, 250 × 4.6 mm i.d. and YMC C_{18} 3 μm, 120 Å, 150 × 4.6 mm i.d.). The applications of Chromolith columns to impurity profiling of drugs substances, monitoring of reaction mixtures, and separation of mother liquors were demonstrated with high resolution as well as high speed. The analysis time were decreased by three to seven times on the monolithic column, while maintaining the comparable resolution to the typical 5 μm particle-packed 250 × 4.6 mm i.d.

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Finally, an overview of some practical aspects and applications of monolithic columns to pharmaceutical analyses has been shown by Wu et al.[19] Wu's conclusion is that monolithic columns offer a new alternative for high speed separations, with the aim of increasing productivity in drug substance process development.

In this work, pressure drop, column efficiency, and retention behavior of monolithic columns (Chromolith type) were evaluated and compared with those of conventional columns packed with porous stationary phase particles. As analytical columns were used: Chromolith SpeedROD RP-18e (50 × 4.6 mm i.d.) and Performance RP-18e columns (100 × 4.6 mm i.d.); YMC-Pack Pro C_{18} (50 × 4.6 mm i.d., 3 μm); Waters Symmetry C_{18} column (50 × 4.6 mm i.d., 3.5 μm); Platinum EPS C_{18} column (50 × 4.6 mm i.d., 1.5 μm); Ace5 C_{18} column (250 × 4.6 mm i.d., 5 μm). The pressure drop for the Chromolith monolithic columns is approximately three times lower than that for the 3.5 μm particle-packed column and nine times lower than that for the 1.5 μm packed columns. Furthermore, the separation on the monolithic column can be performed at high flow rates without significantly sacrificing column efficiency. The selectivity of the C_{18} monolithic column for neutral and basic compounds is comparable to that of conventional packed C_{18} columns, although Chromolith column provides less retention in reversed-phase HPLC.

Seeing that high flow rates and low retention factors are typically observed in fast separations with monolithic columns, the same as using short packed columns, it must be paid special attention to the potential extracolumn band broadening effects in order to obtain the optimum separations results.

Instrumental Requirements

The performance of the fast LC column is strongly influenced by the system configuration, especially with respect to total void system. Considering a given HPLC equipment, the elution volumes are smaller in high speed columns than conventional ones, so the system's contribution to elution volume of analytes is more important for HSLC columns. This situation involves a significant loss in effectiveness for a short column in comparison with a longer conventional one, if extracolumn volume is not reduced. For this reason, some changes are necessary in the standard HPLC set-up to achieve the maximum performance of high speed columns. Basically, the modifications involve the injection system (maximum volume $\leq 3\,\mu L$), the detector cell dead volume ($\leq 3\,\mu L$), the connecting capillary tubes (length $\leq 80\,cm$, inner diameter $\leq 170\,\mu m$) and when injection valve turns (manual or automatically) slowly, it is convenient to install an injector bypass that avoids pressure pulses that could deteriorate short columns quickly.[20] Other instrumental aspects should also be considered in HSLC, such as the time constant detector ($<100\,ms$) to collect data from narrow peaks, and the dwell volume ($<0.25\,mL$) to run fast gradients.

The classical sizing technology is time-consuming and not very efficient per unit of time. Nowadays, commercial HSLC equipments are available. However, from our point of view, the adaptation of our normal HPLC equipments is recommendable since these modifications are simple and economical. Furthermore, the methods need to be conducted on existing instrumentation to minimize capital investment cost.

In order to avoid the chromatographic system adaptation for reducing the instrumental bandwidth, an alternative approach is the use of larger retention volumes obtained by an increase in column diameter (7 mm), which allows the employment of conventional HPLC instruments.[21]

APPLICATIONS

High performance liquid chromatography is widely used in many analysis fields thanks to its great versatility, such as pharmaceuticals, biological fluids, drug discovery, environmental, natural products, food, DNA, proteins, etc. In recent years, the demand to improve the productivity of analyses, as well as to provide enabling information to make better

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real-time processing decisions has significantly raised the level of interest in high speed liquid chromatography.

An in-process control (IPC) is a monitor of reaction progress in the synthesis of a given substance and signals the production chemist to proceed with a subsequent unit operation. When the IPC calls for HPLC as the measurement technique, it is usually because there is a need to monitor disappearance and formation of reaction components. Such an assay would require adequate resolution of the reaction species, sufficient sensitivity to quantitate the dwindling component, and typically, the ability to accommodate various sample loads. Emphasis should be placed on shortening run time, resolution of key components, optimizing sensitivity, and understanding potential interferences by reaction components.[22] An important feature of IPC testing is speed, as the production chemist requires a fast data turnaround. For instance, in pharmaceutical process development, where trials such as reaction monitoring, column fraction screening, analysis of mother liquors and unstable analytes are completed.

Speed in HPLC can be used advantageously if the analysis time is limited by the chromatographic time. This is the case when simple sample preparation possibilities exist or no sample preparation is required as, for example, with drink solutions, injectables and dissolution rate samples.[23]

The number of HSLC applications is not equally distributed between the different analysis fields, due to fast analyses are not so important for all of them. Up to date, most of the efforts of HSLC focus on improving conventional HPLC methods for substituting them in routine quality control, with the aim of higher sample throughput and reduced cost per analysis. For this reason, the applications of HSLC for in-process control are not many and they are only frequent in those fields where faster analyses are essential.

The pharmaceutical industry has traditionally been at the forefront of applying liquid chromatography technology. In fact, 45% of the HPLC market can be found in the pharmaceutical sector, approximately.[24] Some pharmaceuticals applications found in the literature, for in-process control or at least with potential to do it, are mentioned next.

Sotalol, a β adrenergic blocking agent used in the treatment of cardiovascular disorders, and three of its impurities have been separated in less than 90 s by ion-pair HSLC using a C_{18} Pecosphere CR column (33×4.6 mm i.d., 3 μm) at a flow rate of 4 mL min^{-1}.[4] Using a C_{18} DB Supelcosil 5 μm column, these authors also separated didanosine (an antiviral drug) and five potential impurities in less than 80 s. These analysis times are, respectively, about 5 and 10 times lower than those obtained with conventional columns.

Production and downstream processing in biotechnology requires fast and accurate control of each step in the process. Exact data are also needed to satisfy the demands of regulatory authorities with regard to subsequent registration of the product as a drug. For these purposes, Josic et al.[25] have developed and investigated the use of compact, porous disks for fast separation of biopolymers with regard to their performance and speed. The

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construction of separation device containing a compact porous disk with 10 mm diameter and 3 mm layer thickness and its use in the separation of standard proteins obtained by an anion-exchange, cation-exchange or hydrophobic interaction mode were demonstrated. The power of the improved construction of the disk unit is tested on the applications for in-process control during isolation of plasma proteins. Thereby, when α_1-antitrypsin is isolated from human plasma, transferrin and human serum albumin appear as main contaminants to be removed in the subsequent purification process. A statement about the results of the purification process can be made within 40 s using two buffer (10 mM Tris-HCl, pH 7.4 and 1 M NaCl) as mobile phase in gradient mode at 5 mL min^{-1}. To sum up, the authors think that when these separation units are used in the analytical field, the time limit is set not by the speed of the separation itself but by the characteristics of the hardware, above all the dead volume.

Branovic et al.[26] have shown that plasmid DNA can be isolated from bacterial cells using alkaline lysis followed by anion-exchange chromatography on a CIM DEAE disk monolithic column. The purity of the isolated sample was similar when compared to established methods, but the separation was faster. As an additional advantage, a consecutive usage of the same CIM DEAE monolithic column was possible due to a successful regeneration procedure. It was also illustrated that disk monolithic columns can be applied for fast analytical in-process control of the plasmid DNA purity.

Stewart et al.[27] have described a fast HPLC method utilizing non-porous silica (NPS) technology to analyze a three component analgesic mixture. In the development of this method, several NPS columns were examined with 33 mm length and 1.5 μm diameter particles or 100 mm length and 3.0 μm particles available commercially. It was determined that a 100 × 4.6 mm i.d. monomeric non-porous ODS column with end-capping provided the best separation (Rs $>$ 1.5) and peak shapes (lower tailing factors), with a reduction in backpressure and with a short retention time when a 95 : 5 (v/v) aqueous phosphate buffer (pH 3.0)–methanol mobile phase was used. A comparison HPLC run was made on a conventional bare silica column using the official USP compendial method for aspirin, caffeine and codeine and the authors conclude that the non-porous ODS column showed at least equivalent efficiency and an important reduction of analysis time (8.5 vs. 3 min). The need to reduce extracolumn effects in order to achieve optimal results, as well as keeping the amount of organic modifier in the mobile phase within 1% of the specified amount was pointed out.

Other authors have also studied the capability of NPS columns to replace standard ones with the purpose of improving in-process control and drug discovery in the pharmaceutical industry.[28,29] Successful transfers of several procedures are illustrated from 125 or 250 mm columns packed with 5 μm porous stationary phases to 33 mm columns packed with 1.5 μm non-porous particles. In addition, it is demonstrated a fast generic gradient method using a narrow-bore column packed with small NPS particles, and a

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completely automated HPLC workstation for the control analysis of combinatorial libraries that are developed in pharmaceutical research. Thus, a test mixture of 12 compounds with a wide polarity range is separated in 1.5 min with a cycle time of 3.5 minutes. In both cases, important run time reductions were obtained and sample throughput was enhanced considerably.

Hsieh et al.[30] have developed an efficient bioanalytical method based on HPLC-MS/MS for the simultaneous determination of early drug discovery compounds in mouse plasma. The overall goal was to improve the throughput of the rapid pharmacokinetic screening process to shorten the times for drug discovery in pharmaceutical industry. Sample preparation is often the rate-limiting step in developing higher throughput HPLC-MS/MS assay for drugs in biological fluids. Thus, the authors developed a simple direct injection method with one mixed-function column to perform on-line both sample clean-up and chromatographic separation for quantitative multi-component analysis of plasma samples with the sample pooling technique. The column employed was a Capcell MF C_8 (50×4.6 mm i.d., 5 mm) at 1 mL min^{-1} and the performance was examined by using six drug compounds at early discovery stage throughout the experiments. The total run cycle time can be achieved within 4 min per sample.

An HPLC method to determine simultaneously ketoconazole and formaldehyde in an anti-dandruff shampoo, originally developed on a long column, was transferred to two short columns by Heyden et al.[31] Ketoconazole is a broad-spectrum antifungal agent used as the active ingredient in an anti-dandruff shampoo, whereas formaldehyde is a preservative used in many cosmetic products with allowed maximum concentration of 0.2%, because higher levels can harm human health. The only modification of HPLC conditions was the injection volume reduction from 20 μL, for conventional column, to 5 μL for the short high speed ones. The analytes were separated using three different analytical columns: Altima C_8 (250×4.6 mm i.d., 5 μm), Zorbax SB C_8 (75×4.6 mm i.d., 3.5 μm) and Discovery C_8 (50×4.6 mm i.d., 5 μm). The mobile phase contains a mixture 45 : 55 (v/v) of acetonitrile and 0.01 M $NaH_2PO_4 \cdot H_2O$ pH 4.0 at a flow rate of 1 mL min^{-1} and at room temperature. Compared to the long column, the analysis times were reduced with a factor of at least five, for both ketoconazole and formaldehyde. At the same time, a good selectivity and resolution was reached using these short columns. Therefore, the transfer was successful and the obtained assays on the short columns are applicable for fast routine analysis.

Our research group has been working in the development of new HPLC methods for in-process control of new active pharmaceutical ingredients (API) in collaboration with a pharmaceutical company. In the last years, due to the increasing interest of HSLC, we have developed new faster HPLC methods based on high speed columns.

Monitoring the concentrations of API, their precursors and by-products is required for the optimization of their production. This allows the identification of the bottlenecks in their synthesis and is also an essential parameter in their

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scale-up process. During production, the concentrations of the key components are usually monitored on-line and the information used for process control. Therefore, the time lag of the analysis caused by sampling and preconditioning must be short enough to detect the actual state of the reaction, as well as, a short analysis time are preferred to making decisions in real-time.

For in-process control of sulbactam,[32] a β-lactamase inhibitor, a TSK-GEL Super ODS column (50 × 4.6 mm i.d., 2 μm) at 40°C was employed. Sulbactam, its five synthesis precursors and three unknown impurities were separated well in 2.5 min using an aqueous phase of 5 mM tetrabutyl ammonium hydroxide plus 15 mM KCl adjusted to pH 6 and acetonitrile in gradient mode at 1.5 mL min^{-1}. Compared with a conventional column, separation was performance in approximately five times less analysis time. As well, a Kromasil 100 C_{18} microbore column (50 × 2.1 mm i.d., 3.5 μm) at 30°C using 5 mM KH_2PO_4 pH 6.5 and acetonitrile as mobile phase in gradient mode at 0.4 mL min^{-1} was used for in-process control of rifabutin,[33] a spiropiperidyl-rifamycin derivative antibiotic. The analysis time is 1.8 min, so this was a ten-fold reduction in separation time from the assay performed on longer column, dramatically decreasing solvent consumption and the cost per analysis. Both methods were validated in accordance with the International Conference on Harmonization guidelines (ICH) and adequate resolution ($>$1.5) and asymmetry ($<$1.5) were obtained in all cases.

A well-functioning process monitoring system is necessary for the optimization of synthetic and purification processes and to maintain the conditions at the optimum level required to secure production of high purity API with maximum yield. On the basis of our experience, the desired yield and purity can only be reached through improved, expanded analytical control, and fast HPLC can be successfully implemented for routine work.

High resolution and short analysis times are also ideal for checking for precursors or related products and breakdown compounds. Joseph[34] has shown the analysis of clindamycin, an antibiotic, and its precursor, lincomycin, on short columns packed with microparticles. Using an isocratic mode, the analysis time is 9 minutes with Rapid Resolution SB-C_{18} column (50 × 4.6 mm i.d., 3.5 μm) and 6 minutes with Rapid Resolution HT SB-C_{18} column (30 × 4.6 mm i.d., 1.8 μm). But using a gradient, the compounds are resolved in only 1.5 minutes on HT column with re-equilibration in 2 minutes, for a total analysis time of 3.5 minutes.

Li[35] has developed a HSLC method to analyze the extract from the dried flowers of St. John's Wort, an anti-inflammatory agent and anti-depressant used since the Middle Ages. An analysis time of 3 minutes is obtained on Alltima C_{18} Rocket (53 × 7 mm i.d., 3 μm) column at 4 mL min^{-1} flow rate.

Diehl et al employ packed high speed columns, in two different examples, to show that higher sample throughput and reduced costs are possible in pharmaceutical applications. First, a separation of six analytes (caffeine, aniline, *N*-methylaniline, 2-ethylaniline, 4-nitroanisole and *N*-*N*-dimethylaniline) on XTerra MS C_{18} column (150 × 4.6 mm i.d., 5 μm) took place in a

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gradient mode of total cycle time of 25 min at 1.4 mL min^{-1} flow rate. Whereas the scaled down separation on a XTerra MS C_{18} column (20 × 4.6 mm i.d., 3.5 μm) at 3 mL min^{-1} has a total analysis time of 3 min, what represents a 8.3-fold reduction of the cycle time.[36] And second, they also demonstrated a faster method than an official one for analyzing a mixture of caffeine, acetaminophen and aspirin. The USP method is an isocratic run on a C_{18} column 150 mm long. It was transferred from the Symmetry C_{18} column (150 × 3.9 mm i.d., 5 μm) to the Symmetry C_{18} *IS* column (20 × 4.6 mm i.d., 3.5 μm) and resulted in 3-fold run time reduction, from 6 to 2 minutes at 1 and 3 mL min^{-1} flow rate, respectively.[37]

A micellar electrokinetic chromatographic (MECK) method and a fast reversed-phase liquid chromatographic one have been developed by Dolezalová et al.[38] for determining the purity of ampicillin. The experimental results presented in this work clearly show that MEKC and HPLC with a monolithic silica C_{18} stationary phase are the methods of choice for the determination of the purity of ampicillin. As compared with the pharmacopoeial test for related substances, the elaborated MECK and HPLC methods are much faster and consequently offer higher sample throughput. The MECK method has benefits over the HPLC method, which is usual with CE technique: it is easier and reduces running costs. However, at the same time it is less sensitive. A combination of both methods can be very useful in impurity profiling for confirmation of number, identification, and level of impurities. The HPLC method employed a Chromolith Performance RP-18e monolithic column (100 × 4.6 mm i.d.) and a mobile phase composed of phosphate buffer, pH 5.2 and acetonitrile. The flow rate was kept at 4.0 mL min^{-1} and the column temperature was 40°C. The elaborated HPLC method, which used a monolithic silica C_{18} column, proved to be much faster and simultaneously highly efficient, when compared with HPLC methods using traditional particulate columns. As compared with the pharmacopoeial methods, the suggested chromatographic conditions lead to a considerably shortened analysis time (15 vs. 50 min).

In order to evaluate the applicability of a monolithic C_{18}-bonded silica column for the rapid HPLC separation of ingredients in medicinal plants and their phytopharmaceutical preparations Schmidt[39] has proposed a very easy and successful transfer for a HPLC separation method for quality control of *Harpagophytum procumbens* (Devil's Claw), developed on a conventional Hypersil ODS, 125 × 4 mm i.d., 5 μm particle-based silica column to a monolithic silica column. Two monolithic columns, Chromolith Performance RP-18e with the dimensions 100 × 4.6 mm i.d. were coupled in series and comparable results were found to the conventional column with the advantage, that a higher flow rate could be used due to a flat van Deemter curve. The flow rate was increased from 0.8 up to 5 mL min^{-1} without any loss of resolution and the run-time reduced from 30 to 5 min. The author shows that even if you compare the costs of the columns (two Chromolith columns in series are three times as expensive

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as one Hypersil column) the new method is very valuable for commercial quality control where speed of analysis is crucial to free up HPLC systems and analyst time for other projects.

Nováková and Solich[40] have tested two types of columns: 75 × 4.6 mm i.d., 3.5 μm and 50 × 4.6 mm i.d., 1.8 μm; both packed with Zorbax Eclipse XDB-C18, for analysis of estradiol (active substance used for treatment of climacteric syndrome symptoms and postmenopausal osteoporosis), methylparaben and propilparaben (preservatives), estrone (degradation product), and hydrocortizone as internal standard. For the 3.5 μm particle sized column, 2.5 mL min^{-1} were chosen as a maximum flow rate and the compounds were separated in 3.5 min at 40°C, whereas for 1.8 μm particle sized column, the maximum flow rate tolerated by the chromatographic system was 1.2 mL min^{-1} and the compounds were separated in 4.5 min at 40°C. The analysis time, using a 250 × 3 mm i.d., 5 μm column, was 11 min.

A fast and reliable analytical HPLC method has been developed by Solich et al.[41] for determination of indomethacin in pharmaceutical preparations. Indomethacin is a non-steroidal anti-inflammatory, analgesic and antipyretic drug used for the treatment of symptoms of rheumatoid arthritis. In spite of the large number of HPLC methods for indomethacin determination, up to date, there is no method for determining indomethacin and its two degradation products in one analysis simultaneously, including internal standard as well. As many of these methods are time-consuming and impractical for routine analyses or as result of their instrument complexity and the high cost of analysis, a new HPLC method is proposed based on a high speed column with UV detection. While the conventional C_{18} stationary phases were not convenient enough to achieve quick and reliable separation, Zorbax SB-Phenyl analytical column (7.5 × 4.6 mm i.d., 3.5 μm) enables separation of indomethacin and its two degradation products (4-clorobenzoic acid and 5-methoxy-2-methylindoleacetic acid) in 7.5 min. Chromatography was performed using isocratic elution with binary mobile phase composed of acetonitrile and 0.2% phosphoric acid (50:50) (v/v) at flow rate 0.6 mL min^{-1}. This method has been used for quality control in the manufacturing process and degradation process control in stability studies of indomethacin formulations.

Although, there are not many applications of HSLC for in-process control of proteins, the appropriate HPLC technology is available currently. Some outstanding examples of these new advances are cited next.

Some years ago, Horváth[42] and Kourganov[43] showed fast proteins and peptide separations on high speed columns based on silica with porous and non-porous stationary phases. Now, they can also be completed on polymeric based columns, both packed short and monolithic, and microbore and capillary columns are available, too. Various pore sizes are available using 3 μm polymeric particles, for example 100 Å for peptide analysis and 300 Å with improved mass transfer characteristics for large polypeptides and globular proteins: PLRP-S 100 Å and 300 Å stationary phases, available in a 50 mm column length and 4.6, 2.1 and 1.0 mm i.d.[44] Also, it has been

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described the use of 1.5 μm fully porous media combined with 500 Å pore size for fast protein analysis: Alltech Prophere HP ZAP C_{18} (10 × 4.6 mm i.d., 500 Å, 1.5 μm).[45] This approach delivers the speed, loading capacity, and resolution high-throughput protein separations require. Finally, monolithic capillary columns (50 × 0.2 mm i.d.) consisting of PS-DVB (polystyrene-divinylbenzene polymer) at 2.5 μL/min flow rate have also been used for protein and peptide separations in HPLC-MS.[46]

On the other hand, to facilitate the protein precipitation as sample preparation method, new protein precipitation procedure has been developed. A 96-well plate contains a stack of membranes for filtration, which maximize recoveries and eliminate cloudy filtrates. This method eliminates time-consuming and extra sample-handing steps. So, once the samples are processed, a fast method of HPLC-MS-MS can be run utilizing microbore HPLC column. In this way, combining fast and simple protein precipitation enabled by the Sirocco Protein Precipitation Plate with XTerra MS C_{18} *IS* (20 × 2.1 mm, 3.5 μm) columns provides analysts with improved sample preparation and analysis of bioanalytical samples.[47]

The use of short monolithic columns results in a very fast analysis in typically 10-15 min for protein digests, with peak capacities of up to 150 peaks. Beside the analysis of peptides, polypeptides and protein digests there is an increasing interest for the analysis of intact proteins in Proteomics. Such fast and high resolution separations make these columns ideally suited for on-line coupling to ESI-MS and off-line LC/MALDI target preparation for MS proteomics analysis.[48]

As happens in other fields, run times of food and beverage HPLC methods can be quite long when using conventional (150 × 4.6 mm i.d. or 250 × 4.6 mm i.d.) columns, on the order of 15–30 min. Furthermore, it should also be taken account tedious and time-consuming sample pre-treatment are usually necessary. Such long analyses severely limit productivity in a growth industry where high sample throughput is paramount. Some attempts of HSLC in food analysis are indicated next. Profiling carbohydrates in orange juices and drinks has been completed on Alltech Prevail Carbohydrate ES column (53 × 7 mm i.d., 5 μm) at 1.3 mL min^{-1} in 7 min, so as many as eight samples per hour could be analyzed for the simple sugars.[49] This technique can be used for routine quality monitoring and determining contamination or adulteration.

A fast and reliable method for the determination of polyphenolic ingredients in red wine has been applied by Lamotte.[50] The wine is only filtered through a 0.2 μm filter and injected directly on the column without dilution. The analytes are separated in an isocratic mode on a standard HPLC device within 4 minutes at 2.5 mL min^{-1}, using a ProntoPEARL $TPP_{sub2}C_8$ ace-EPS column (50 × 4.6 mm i.d., 1.8 μm).

The determination of the isoflavones in soy samples, phytotherapeutical preparations and nutritional supplements, is generally performed on conventional particulate silica or polymer-based RP C_{18} columns. Owing the

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substantial difference in the concentration range of isoflavone aglycones and glycosides, two HPLC runs are necessary to determine the total amount of isoflavones and analysing samples in duplicate leads to a total run time of about 4 h. To overcome this problem, Apers et al.[51] have developed an HPLC method using monolithic silica-based RP C_{18} columns. In order to speed up analysis and to achieve a good separation of the late-eluting isoflavones, i.e., acetyldaidzin, acetylglycitin, daidzein, glycitein and acetylgenistin, two Chromolith Performance RP-18e (100 $\times$ 4.6 mm i.d.) columns were linked. The analysis was speeded up by applying higher flow rates with parallel adaptation of the mobile phase gradient applied on the particulate column. A spectacular decrease of the analysis time, almost three-fold, was achieved by applying a flow rate of 3–4 mL min^{-1} without loosing any separation efficiency.

As can be seen in all these examples, there is an increasing interest in the analysis time reduction and the improvement of conventional HPLC methods used for in-process and quality control. Obviously, the practical needs lead this evolution, and today HSLC mainly focus on pharmaceutical and bioanalytical applications.

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CONCLUSIONS AND PERSPECTIVES

High speed liquid chromatography represents a significant advance on HPLC theme because improves the in-process control methods for applications where speed is essential (pharmacokinetic studies, reaction monitoring, unstable analytes tests, investigation of fast kinetics, etc), as well as reduces operational costs and increases sample throughput analysis, in general, for all kind of HPLC applications. Therefore, HSLC let us control the manufacturing processes more efficiently and increase industrial productivity.

High speed columns, both packed short and monolithic columns, are the most popular approaches to fast separations and, now, they are a real alternative to standard ones because offer higher effectiveness with lower functioning costs.

Column technology has been the main responsibility for the advances in this field, basically for reverse phase. Indeed, it is the most versatile mode in HPLC. So, the development of new stationary phases with other separation mechanisms may further expand the range of samples amenable to analysis, achieving a HSLC expansion to other applications, such as chiral, ion-exchange and bioaffinity separations.

An increasing number of papers about HSLC for in-process control is observed in the scientific literature. However, they are still not many in comparison with those of HPLC. Nevertheless, an important development of this methodology is expected due to its great potential.

Finally, it should not be forgotten that one of the more interesting areas of separation science is currently the move towards miniaturization. In this sense, the development of capillary and nanobore high speed columns also contribute

to improve the analyses in those application fields where mass spectrometry detection is necessary. For example, drug discovery, biological samples, natural products, proteins, etc.

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