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REVIEW

New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches

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Abstract Recent developments in chromatographic supports and instrumentation for liquid chromatography (LC) are enabling rapid and highly efficient separations. Various analytical strategies have been proposed, for example the use of silica-based monolithic supports, elevated mobile phase temperatures, and columns packed with sub-3 μm superficially porous particles (fused core) or with sub-2 μm porous particles for use in ultra-high-pressure LC (UHPLC). The purpose of this review is to describe and compare these approaches in terms of throughput and resolving power, using kinetic data gathered for compounds with molecular weights ranging between 200 and 1300 $g mol^{-1}$ in isocratic and gradient modes. This study demonstrates that the best analytical strategy should be selected on the basis of the analytical problem (e.g., isocratic vs. gradient, throughput vs. efficiency) and the properties of the analyte. UHPLC and fused-core technologies are quite promising for small-molecular-weight compounds, but increasing the mobile phase temperature is useful for larger molecules, for example peptides.

Keywords UHPLC · UPLC · HTLC · Monolith · Fused-core · Kinetic plots

Introduction

High-performance liquid chromatography (HPLC) is a well-established separation technique that can be used to solve numerous analytical problems. During the last few years substantial improvements, for example innovative supports and advanced instrumentation, have been brought to conventional HPLC, enabling faster analyses and higher separation efficiencies [1, 2]. Such advances were mainly driven by the need to cope with either a growing number of analyses or with more complex samples.

There is a growing demand for high-throughput separations in numerous fields, including toxicology, clinical chemistry, forensics, doping, and environmental analyses, where the response time must be reduced. The pharmaceutical field, with its need for enhanced productivity and reduced costs, is the main driving force for faster separations [3]. Because of the large number of analyses required for common pharmaceutical applications, for example purity assays, pharmacokinetic studies, and quality control, rapid analytical procedures (less than 5 min, including equilibration time) are mandatory [4].

Highly efficient separations are also necessary for many applications, including genomics, proteomics, and metabolomics, all of which deal with very complex samples, such as biological samples, tryptic digests, or natural plant extracts [5, 6]. With such difficult samples, conventional HPLC systems have some obvious limitations, thus demanding analytical procedures to yield high resolution within an acceptable analysis time, even when a large number of compounds need to be separated.

The purpose of this review is to guide the separation scientist in selecting the most appropriate analytical system among the new techniques recently launched. For this purpose, the different commercialized approaches are first described and compared in terms of both throughput and resolving power, using kinetic data gathered for compounds with a variety of chemical diversity and molecular weights ranging from 200 to 1300 $g mol^{-1}$ in both isocratic and gradient modes.

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Brief presentation of strategies and main features

Over the last decade, several approaches based on the use of monolithic supports, high-temperature liquid chromatography (HTLC), fused-core technology, or columns packed with sub-2 μm particles under ultra-high-pressure conditions (UHPLC), have been developed and commercialized to improve throughput and efficiency in LC [7].

Monoliths

Monolithic supports consist of a single rod of porous material with several unique features in terms of permeability and efficiency. These materials were originally developed by Hjerten et al. [8], Svec and Frechet [9], and Tanaka, Nakanishi and co-workers [10] during the 1990s. Various types of inorganic (e.g., silica, zirconia [11], carbon [12], and titania [13]) and organic (e.g., polymethacrylate, poly(styrene–divinylbenzene), and polyacrylamide [14]) monoliths can be prepared, but only polymethacrylate, poly(styrene–divinylbenzene), and silica-based monolithic columns are commercially available. The organic monoliths are of only limited interest in conventional HPLC but are useful for separation of macromolecules, for example proteins or antibodies [15, 16], or for a wider range of molecules when using capillary electrochromatography (CEC) [17]. Silica-based monolithic supports have been available from Merck and Phenomenex since 2000, under the trademarks Chromolith and Onyx, respectively, and remain the most widely used in HPLC. The bimodal structure of silica-based monoliths is characterized by 2-μm macropores and 13-nm mesopores, leading to efficiencies similar to that of porous silica particles (3–3.5 μm) [18]. Tallarek et al. demonstrated that monolithic silica columns have elevated permeabilities, equivalent to a column packed with 11-μm particles [19]. Furthermore, the size of macro and mesopores can be independently controlled through the sol-gel synthesis process to tune permeability and chromatographic efficiency. The second-generation commercial silica monoliths will certainly benefit from this feature [18].

The low backpressure generated and good mass transfer enable use of elevated flow rates (3 to 10 times larger) when working with a conventional column length, thus enabling ultra-fast separations, down to only a few seconds for separation of several substrates and metabolites [20]. Alternatively, it is also possible to use very long monoliths at a reasonable flow rate to achieve elevated resolution in a practical analysis time. For example, Tanaka et al. [21] constructed an 11.4-m column by coupling numerous monolith columns. As shown in Fig. 1, this arrangement provided 1,000,000 theoretical plates for an analysis time of approximately 16 h.

Despite these outstanding properties, monoliths are not widely used and to date, less than 1% of chromatographers routinely use silica-based monolithic columns [22]. Several explanations for their limited use include patent exclusivity, which leads to a limited number of suppliers, column chemistry and geometry (columns are now available in 2, 3, and 4.6-mm I.D. but with a maximum length of only 100 mm), and the limited resistance of the support in terms of pH [23] and, more importantly, backpressure (ΔP_{max}=200 bar).

High-temperature liquid chromatography (HTLC)

An elevated mobile phase temperature ($60<T<200$°C) is valuable for improving chromatographic performance. A temperature increase results in a significant reduction of mobile phase viscosity, η, leading to higher diffusion coefficients for the compounds and improved mass transfer, therefore increasing the optimum linear velocity (u_{opt} is proportional to T/η) [24–26]. Temperature, which is directly proportional to solvent viscosity, also causes a significant reduction in column backpressure with use of a constant flow rate. Because of these properties, it is possible to maintain resolution and increase the speed of separations by a factor of 3 to 5 (90 °C), and up to a factor of 20 (200 °C), with methanol as the organic solvent [27]. Alternatively, longer columns with acceptable backpressure can be employed at elevated temperatures, although it becomes difficult to work under optimum flow rate conditions. For instance, Sandra et al. experimentally demonstrated the possibility of reaching efficiencies higher than 100,000 plates in only 50 min by using 90-cm long columns packed with 5-μm particles at 80 °C [28].

In addition to its kinetic performance, HTLC has some additional advantages, which are summarized in Table 1.

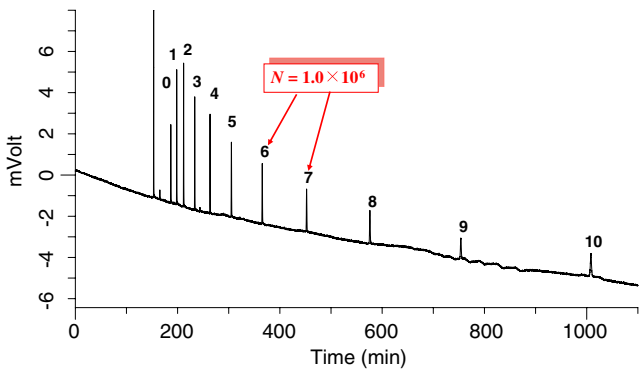

Fig. 1 Highly efficient separation of alkylbenzenes using a long monolithic silica capillary column. Mobile phase: acetonitrile-water (80:20). Column: three monolithic silica C_{18} columns connected in series. Effective length, 1140 cm; ΔP, 35.4 MPa; u, 1.24 mm s^{-1}; detection, 210 nm; temperature, 30 °C. Sample: thiourea and alkylbenzenes ($C_6H_5C_nH_{2n+1}$, n=0–10). Adapted from Ref. [21], with permission

Table 1 Advantages and drawbacks of approaches for high-throughput and high-resolution experiments in LC

Approach	Advantages	Drawbacks
Monoliths	Very low backpressure because of the elevated permeability	Lack of chemistries (C_{18}, C_{18} endcapped, C_8) and providers
	Approach compatible with a conventional HPLC system	Direct method transfer impossible between conventional HPLC and monolithic supports
	Different geometries (e.g. 2.1 mm I.D.) are available	Limited resistance in terms of backpressure (<200 bar) and pH (2<pH<8)
HTLC	Green chemistry: decrease of the organic modifier amount at elevated temperature	Stability of the solutes and silica-based stationary phases can be critical at T>100 °C
	Improvement of peak shape for basic drugs and large molecules (e.g. peptides)	Need to use dedicated instrumentation (preheating and cooling devices + backpressure regulator)
	Possible to use this strategy in conjunction with UHPLC to further improve performance	Method transfer difficult because of changes in selectivity with temperature
UHPLC	Easy method transfer between HPLC and UHPLC	Need to use a dedicated instrumentation (low σ^2_{ext}, elevated acquisition rate, fast injection)
	Important decrease in analysis time	Cost of instrumentation and consumables higher than for conventional HPLC
	Large variety of columns packed with sub-2 μm particles (more than ten providers)	Solvent compressibility and frictional heating are issues for ΔP close to 1000 bar
Fused-Core	Interesting approach to limit diffusion of large molecules in pores	Lack of chemistries (C_{18}, C_8, HILIC) and providers
	The quality of the packing is excellent (h≈1.5) compared with other materials (h≈2–2.5)	Retention and loading capacity slightly lower than conventional HPLC (heart of the particle non porous)
	Approach potentially compatible with a conventional HPLC system	Lower resistance in terms of backpressure (<600 bar) and pH (2<pH<9) compared with UHPLC

First, at elevated temperature, both the dielectric constant and surface tension of water decrease, such that water can replace a large proportion of the organic solvent in the mobile phase (5–10% less organic solvent for each 30 °C change) [29, 30]. In some extreme cases, superheated water has been considered as a suitable mobile phase to separate various steroids at 200 °C [31] or for various other applications [32–35]. Second, improvement of peak shape has been reported for basic compounds, because of a decrease of one pK_a unit for each 30 °C change, and thus a reduction of secondary interactions with residual silanol groups [36, 37]. For large molecules, for example peptides, the peak shape can also be improved, because diffusion coefficients strongly increase with temperature. Finally, temperature can be used to tune selectivity during method development, because of interaction differences between the analyte and the stationary phase with different temperatures [38].

Although HTLC has been investigated in academic laboratories, it remains rarely used in industry. The major constraints of this technique are related to the relatively limited number of stable stationary phases compatible with elevated temperature [39], the required modification of LC equipment to control the mobile phase temperature adequately [40], and, most importantly, the putative thermal degradation of compounds [41]. These factors need to be further investigated.

Ultra-high pressure liquid chromatography (UHPLC)

In LC it is well established that packing columns with small particles results in simultaneous improvement of efficiency, optimum velocity, and mass transfer, as demonstrated in the early development of chromatography [42–45]. Column manufacturers have made many advances in packing materials; since 2004, columns packed with sub-2 μm porous particles have become commercially available and yield reliable performance in comparison to those with conventional particle sizes [46, 47]. However, the pressure required for percolating the mobile phase through a column packed with such small particles can be prohibitive for standard HPLC hardware (ΔP is proportional to d_p^3 at u_{opt}, according to Darcy's law). Some improvements of the chromatographic system have been made to address this problem, and instrumentation that can withstand pressure beyond 400 bar has been commercialized [48]. The term ultra-high-pressure liquid chromatography (UHPLC) is often used to define the use of columns packed with sub-2 μm particles with a higher backpressure requirement (ΔP> 400 bar) [49]. Significant developments in UHPLC technology were recently observed, and currently there is a wide variety of stationary phases packed with sub-2 μm particles (more than 10 providers and 80 chemistries) [50] and

UHPLC instruments (around 10 providers of systems with maximum pressures ranging between 600 and 1,200 bar) [48].

It is theoretically possible to speed up UHPLC separation compared with conventional HPLC while maintaining identical performance [51–53]. For example, Fig. 2 shows a complex separation of 12 compounds carried out in gradient mode with a conventional 150-mm column packed with 5-µm particles. By applying the appropriate chromatographic changes [54, 55], this separation can be transferred to a 50-mm column packed with 1.7-µm particles. The analysis time was reduced by a factor of 9 from HPLC to UHPLC (27 vs. 3 min, respectively), without a loss in peak capacity or change in selectivity. Because it is possible to work up to 1,000 bar with UHPLC instrumentation and because mass transfer is improved with small particles, the highest possible mobile phase flow rate was applied in UHPLC, leading to a 17-fold increase in throughput compared with conventional HPLC (27 vs. 1.6 min, respectively) and with resolution that remained at least equal to 1.5.

Longer columns packed with small particles can also be selected to increase resolution further, even though the optimum mobile phase flow rate cannot always be reached [56, 57]. Finally, as demonstrated in Fig. 2, one of the main features of UHPLC is the possibility of geometrically

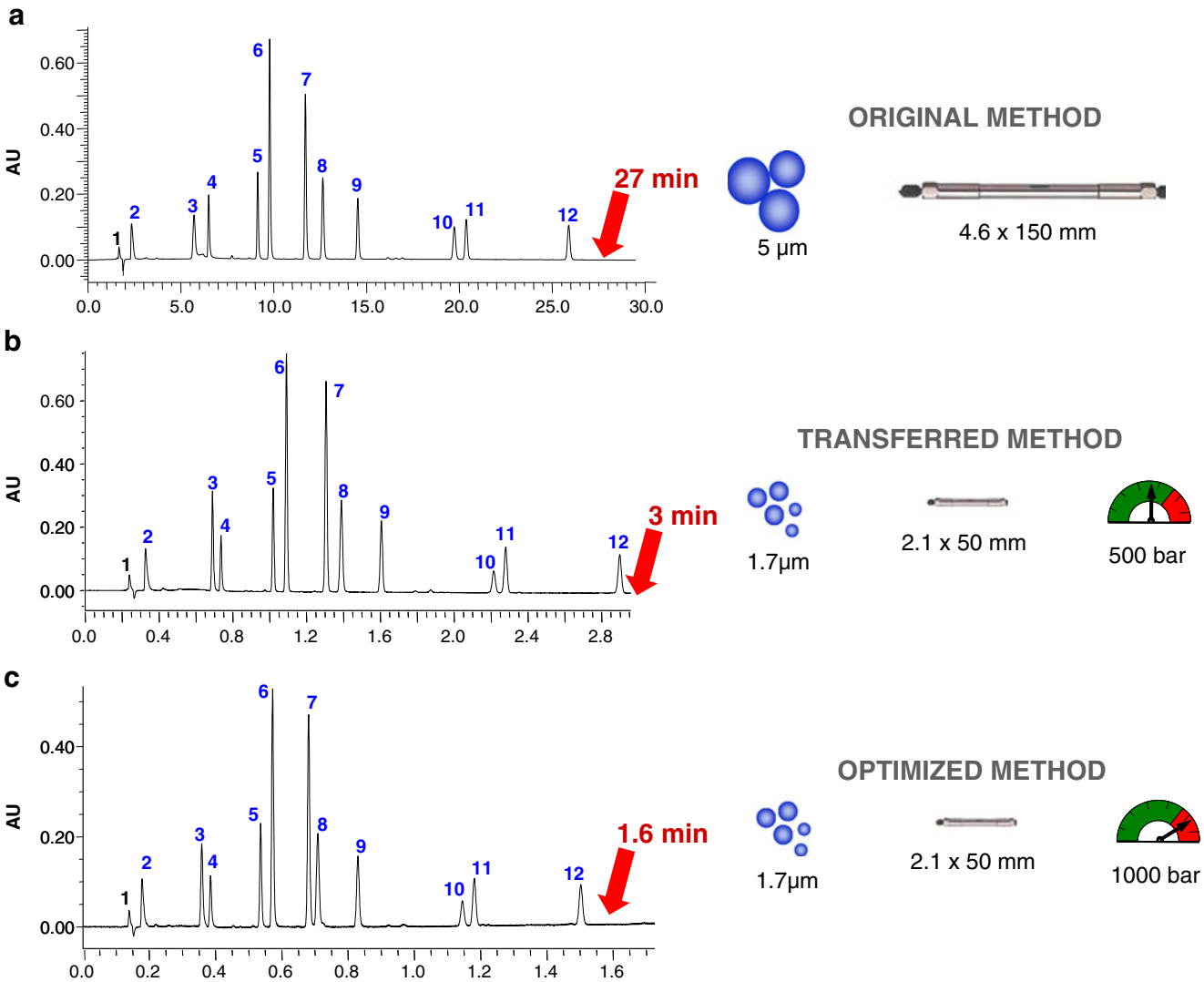

Fig. 2 Method transfer from conventional HPLC to UHPLC. Separation of a pharmaceutical formulation containing the main product (6) and eleven impurities in gradient mode with HPLC and UHPLC systems: (**a**) original HPLC method: column, XBridge C_{18} 150×4.6 mm, 5 µm; flow rate, 1000 µL min^{-1}; injected volume, 20 µL; total gradient time, 45 min. (**b**) Transferred UHPLC method: column, Acquity BEH C_{18} 50×2.1 mm, 1.7 µm; flow rate, 610 µL min^{-1}; injected volume, 1.4 µL; total gradient time, 5.1 min. (**c**) Transferred and optimized UHPLC method: column, Acquity BEH C_{18} 50×2.1 mm, 1.7 µm; flow rate, 1000 µL min^{-1}; injected volume, 1.4 µL; total gradient time, 3.1 min. Adapted from Ref. [55], with permission

transferring existing methods from conventional HPLC, using basic equations of chromatography in isocratic and gradient modes [54, 55], assuming the stationary phase chemistry is available in various particle sizes. This is of prime importance in the pharmaceutical field, because only a partial validation procedure is required when selectivity is maintained [58].

However, drawbacks of UHPLC are:

1. the need to acquire a dedicated system, optimized in terms of high backpressure pumps and an injector, acquisition rate of the detector, injection cycle time, dwell volume and system dead volume [59]; and
2. the frictional heating generated by the elevated backpressure, which produces temperature gradients inside the column [60–65]. This heating problem, which is particularly critical for 4.6-mm I.D. columns and/or when the pressure is close to or higher than 1,000 bar, can be resolved by reducing the column I.D. to 2.1 or 1 mm [66].

Fused-core technology

The most recently reported strategy for improving chromatographic performance is fused-core technology, which became commercially available in 2007 under the trademarks Halo from Advanced Materials Technology (Wilmington, Delaware, USA), Ascentis from Supelco (Bellefonte, Pennsylvania, USA), and, more recently, Kinetex from Phenomenex (Torrance, California, USA). This technology was originally developed by Kirkland in the 1990s to limit diffusion of macromolecules into the pores [77], and smaller particle sizes have now been incorporated to meet current requirements.

This approach generally consists of using 2.7-μm superficially porous particles composed of a 1.7-μm solid inner core and a 0.5-μm porous outer core; Phenomenex recently introduced 1.7-μm particles made of a 1.25-μm solid core surrounded by a 0.23-μm porous shell. In comparison with totally porous particles of similar diameters, the diffusion path is much shorter, because the inner core is solid fused silica, which is impenetrable by analytes (thus decreasing the resistance to mass transfer, the *C* term of the Van Deemter curve) [67, 68]. This tends to limit the axial dispersion of solutes and minimize peak broadening, especially at elevated linear velocities [69]. This characteristic is especially important for the separation of large molecules, for example peptides or intact proteins, where slow mass transfer induces a loss of efficiency with rapid separations on porous particles [70]. Additionally, this material has an exceptionally narrow particle size distribution and high packing density compared with porous particles, leading to a smaller *A* term in the Van Deemter curve (i.e. eddy diffusion) [70]. Indeed, various authors have determined *h* values down to 1.5 for such columns in contrast to values of 2–2.5 for columns packed with porous particles [70]. This type of support can provide speed and efficiency similar to columns packed with sub-2 μm particles. For an identical column length, the semi-porous particles maintain almost the same efficiency as that from sub-2 μm particles but with reduced backpressure [71, 72]. Because of the lower backpressure, longer columns can be considered for increasing the resolving power [73–75].

This approach is being developed rapidly to improve chromatographic performance, because there is no need to update instrumentation for UHPLC. Similar to monoliths, because of patent reasons, the number of suppliers of fused-core particles is still restricted. In addition, a limited number of support chemistries are available, but this is likely to change in the future as these products become more widely accepted [22, 76]. Finally, even if the backpressure generated is a factor of two less than that of columns packed with sub-2 μm particles, the resistance of the support to pressure is also almost a factor of two lower (600 vs. 1,000 bar).

Evaluation of existing approaches

As discussed in the previous section, various strategies have been proposed to improve chromatographic perfor-

Table 2 Description of the LC strategies reported in Figs. 3, 5, and 7

Strategy	Column	Particle size (μm)	Temperature (°C)	ΔP max (bar)
HPLC	Waters Xbridge C_{18}, 50×2.1 mm	5	30	400
HPLC, 1000 bar	Waters Xbridge C_{18}, 50×2.1 mm	5	30	1000
HTLC	Waters Xbridge C_{18}, 50×2.1 mm	5	90	400
Sub-2 μm	Waters Acquity BEH C_{18}, 50×2.1 mm	1.7	30	400
UHPLC	Waters Acquity BEH C_{18}, 50×2.1 mm	1.7	30	1000
HT-UHPLC	Waters Acquity BEH C_{18}, 50×2.1 mm	1.7	90	1000
Fused-core	Supelco Ascentis C_{18}, 50×2.1 mm	2.7 (SPP)	30	600
Monoliths	Phenomenex Onyx C_{18}, 50×2.1 mm	–	30	200

mance. However, it is difficult for a chromatographer to select the most appropriate analytical system among the existing ones. The different approaches are presented in Tables 1 and 2 and compared using optimum experimental data for compounds with molecular weights ranging between 200 and 1,300 $g mol^{-1}$ in isocratic and gradient modes. The three model compounds are butylparaben, a preservative widely used in the pharmaceutical field, with a MW of about 200 $g mol^{-1}$, rutin, a flavonoid glycoside present in various plant extracts, with a MW of about 600 $g mol^{-1}$, and triptorelin, a peptide with a size equivalent to peptides present in tryptic digest, with a MW of about 1300 $g mol^{-1}$. To obtain the data presented in Figs. 3, 5 and 7, we used the kinetic plot method, a well-established strategy for evaluating chromatographic performance in terms of analysis speed, resolving power, and backpressure [78–80].

Separation of small molecules in isocratic mode

In isocratic mode, two important properties were selected to evaluate the throughput and resolving power:

For separation speed, the dead time ($t_{10,000}$) required to attain an efficiency of 10,000 plates was calculated. Such a plate number is generally sufficient for high-throughput separations of conventional samples with a limited number of analytes.

For resolving power, the maximum achievable efficiency $N_{30\ min}$ with a column dead time of 30 min (equivalent to an analysis time of 3 h for k= 5) was considered. This analysis time is quite long but not prohibitive when dealing with very complex samples.

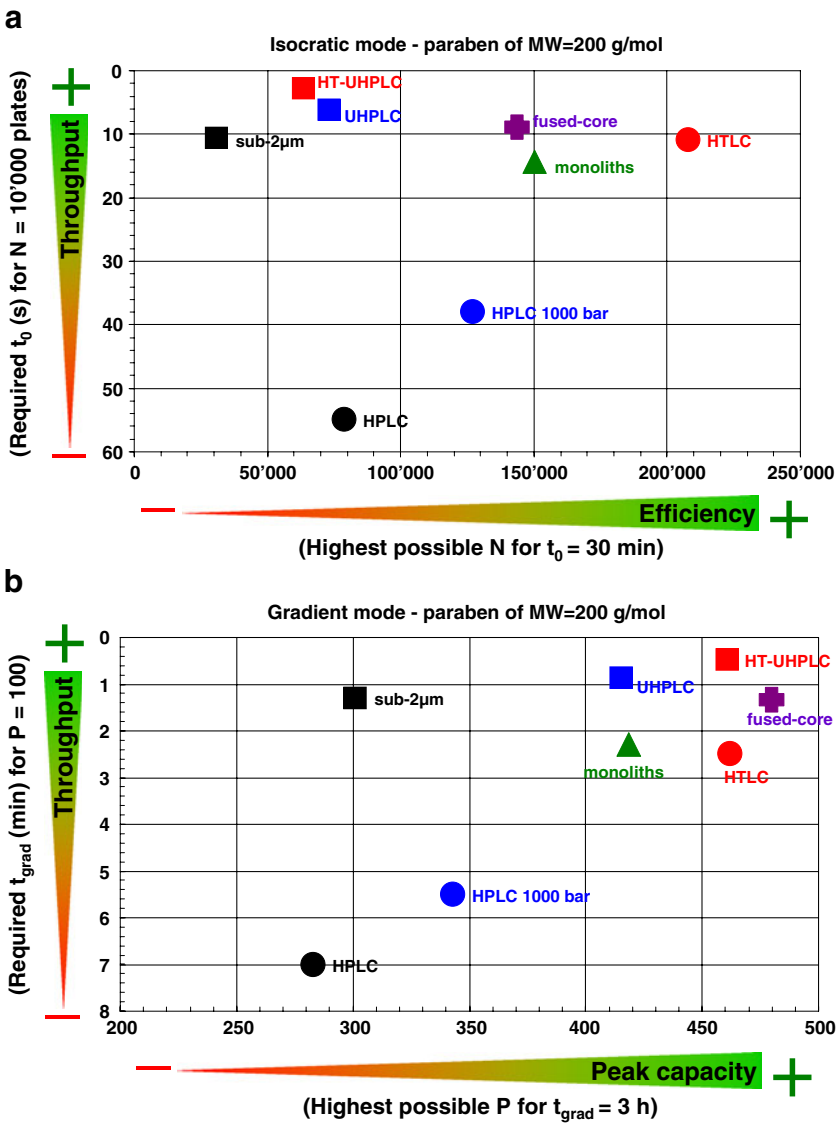

Fig. 3 Performance comparison of LC strategies in terms of throughput and maximum resolution for a model compound, butylparaben, with MW of 200 $g mol^{-1}$. (**a**) Isocratic comparison in terms of throughput (t_0 for N=10,000 plates) and maximum efficiency (N_{max} for t_0=30 min). The data were gathered using the kinetic plot methodology, as described in the text, considering a maximum pressure of 200 bar for monoliths, 400 bar for HPLC, HTLC, and sub-2 µm, 600 bar for fused-core, and 1000 bar for UHPLC, HT-UHPLC, and HPLC. (**b**) Gradient comparison in terms of throughput (t_{grad} for P=100) and maximum peak capacity (P_{max} for t_{grad}=3 h). The data were gathered using the kinetic plot methodology applied to gradient elution, as described in the text and considering the same maximum pressure as for isocratic mode

The values of $t_{10,000}$ and $N_{30\ min}$ were calculated using Van Deemter data (H, u) and permeability values $K_{v,0}$ experimentally determined for each analytical strategy. Then, the data were computed, in agreement with the maximum pressure drop of the system indicated in Table 2, to calculate the column length and mobile phase flow rate required. Thus, the column dead times and plate numbers presented in Fig. 3a always correspond to a pressure drop equal to ΔP_{max} (from Table 2) with variable column lengths and mobile phase flow rates. For a better understanding of the kinetic plot methodology, readers can refer to a few didactic papers [81–84].

Figure 3a shows the results obtained for butylparaben (MW=200 gmol^{-1}). In terms of throughput (Y-axis), HPLC and HPLC 1000 bar ($t_{10,000}$=55–38 s for N=10,000 plates) are clearly not competitive with the other strategies. Although columns packed with 5-μm particles and able to work up to 1,000 bar are not yet commercially available, they were found to be promising and therefore included in this study [85–87]. As expected from theory, monoliths significantly reduce the analysis time ($t_{10,000}$=15 s) because of the low backpressure generated, but they still suffer from higher H values (H=~8 μm, corresponding to 120,000 plates m^{-1}) compared to fused-core or columns packed with sub-2 μm particles (H=~4–5 μm, corresponding to more than 200,000 plates m^{-1}). Methods using HTLC appeared beneficial for reducing the analysis time ($t_{10,000}$=11 s) because of the improvement of diffusion coefficients related to the decrease of mobile phase viscosity with temperature. For example, Yang et al. [88] showed that ultra-fast separations could be achieved at very high temperatures. The authors used an experimental set-up allowing for a flow rate and temperature as high as 15 mL min^{-1} and 150°C, respectively. Separation of five alkylphenones was carried out with a conventional 50×4.6 mm column packed with 2.5-μm particles in only 20 s (ΔP of 360 bar) instead of 20 min at ambient temperature. The use of columns packed with sub-2 μm particles remains the most efficient strategy for reducing analysis time, particularly when small particles are combined with high pressure and elevated temperature, where a 20-fold increase of throughput compared with that of conventional HPLC ($t_{10,000}$=3 s for N=10,000 plates) is possible. As shown in Fig. 4a, a mixture of four preservatives was separated in about 9 s by HT-UHPLC using a 50×2.1 mm, 1.7-μm column at 1.8 mL min^{-1} and 90°C [89].

The maximum efficiency that can be achieved with t_0= 30 min (X-axis, $N_{30\ min}$) is between 31,000 and 208,000 plates. Unfortunately, long columns operating in the B term-dominated region of the Van Deemter curve are required (low mobile phase flow rate) [90, 91]. To reach such efficiencies, we calculated that the column length should be between 0.6 and 3.4 m, and the mobile phase flow rates should range between 50 and 270 μL min^{-1} for a 2.1-mm I.D. column. Therefore, the strategies involving small particles (i.e., sub-2 μm, UHPLC and HT-UHPLC) are less potent (31,000<$N_{30\ min}$<73.000 plates) because of the elevated backpressure generated, thereby limiting the column length that can be employed. This observation has been experimentally confirmed by Sandra et al. [92] who demonstrated that an N_{max} of 74,000 was possible for a test mixture under isocratic conditions at 40°C, using a 450-mm column length packed with 1.7-μm particles at 1000 bar. In

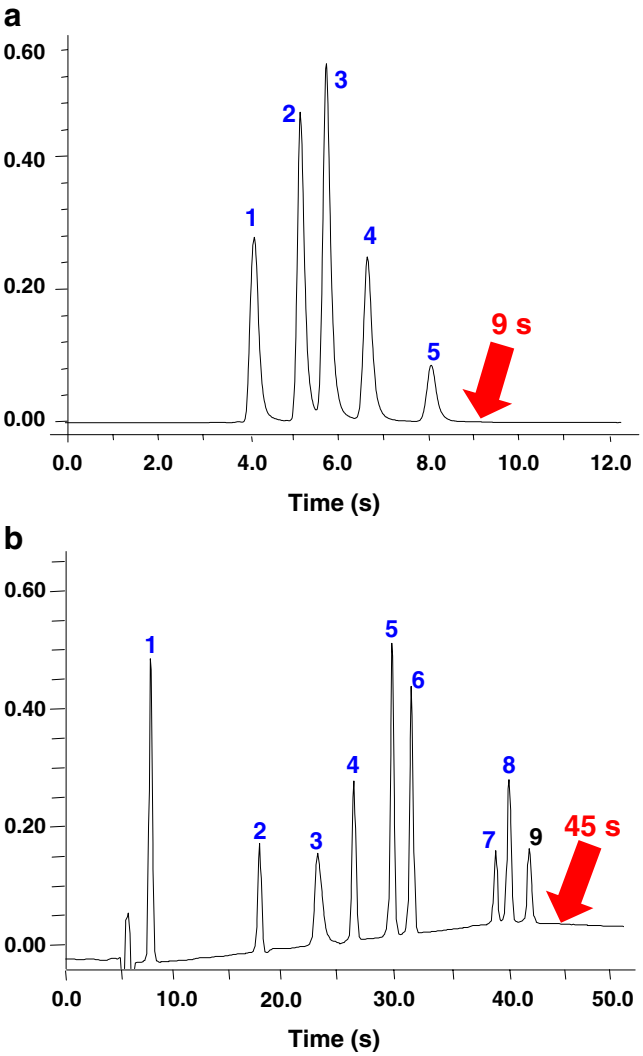

Fig. 4 Ultra fast separations carried out in HT-UHPLC. (**a**) Isocratic separation of various preservatives and uracil. Column, Acquity BEH C_{18} (50×2.1 mm I.D., 1.7 μm); mobile phase, water-acetonitrile (50:50%, v/v); flow-rate, 1800 μL min^{-1}; temperature, 90°C. Compounds: *1*, uracil; *2*, methylparaben; *3*, ethylparaben; *4*, propylparaben; *5*, butylparaben. (**b**) Gradient separation of several doping agents. Column, Acquity BEH Shield RP18 (50×2.1 mm I.D., 1.7 μm); mobile phase, 0.1% formic acid in water-0.1% formic acid in acetonitrile; flow-rate, 1800 μL min^{-1}; temperature, 90°C. Compounds: *1*, acetazolamide; *2*, chlortalidone; *3*, clopamide; *4*, dexamethasone; *5*, furosemide; *6*, indapamide; *7*, bumetanide; *8*, probenecid; *9*, ethacrynic acid. Adapted from ref. [89], with permission

contrast, the maximum efficiency of columns packed with 5-μm particles is around 80,000 plates and can be increased by 60% for ΔP_{max}=1000 bar and by 2.6 fold at elevated temperatures. Because of their elevated permeability, monolithic supports also serve as a good strategy for increasing the plate count compared with conventional HPLC ($N_{30\ min}$ is twofold higher) [93]. However, the column length needs to be around 1.7 m, so 17 of the commercially available 100-mm columns length would need to be coupled in series. Although some authors have coupled up to 10 columns, the cost becomes rapidly prohibitive [94]. Despite the elevated maximum efficiency observed with monoliths, this approach is not competitive with HTLC, because the lower maximum backpressure capability (ΔP_{max}=200 bar) limits the monolith length that can be employed. In HTLC, more than 200,000 plates for a t_0=30 min can be attained, but with a 3 m column, which is expensive (twelve 250-mm columns). Experimentally, Sandra et al. coupled in series eight 250-mm columns packed with 5-μm particles, corresponding to a total length of 2 m. With this configuration, efficiencies as high as 180,000 plates were achieved at 80°C for a test mixture, with t_0 values of 20 min and analysis times around 100 min [95, 96]. Finally, fused-core technology provides $N_{30\ min}$ values similar to those of monoliths, but with more acceptable column lengths and mobile phase flow rates, such as 1 m and 90 μL min^{-1} for a 2.1-mm I.D. column. These results can be attributed to the elevated plates per metre values of the fused-core column in conjunction with backpressure 2 to 3 times lower than for columns packed with sub-2 μm particles.

In conclusion, high-throughput separations require the use of columns packed with small particles and should be carried out at elevated temperature or with highly porous material, for example monoliths, in close agreement with other studies also performed in the isocratic mode [23, 91, 97, 98]. We also demonstrated that temperature and maximum system pressure drop should be increased as much as possible, because both conditions are beneficial for increasing the plate count and throughput.

Separation of small molecules in gradient mode

In complex analysis, for example bioanalysis, impurity profiling, natural product extracts, and peptide mapping, the separation is performed in gradient mode to handle compounds with very different physicochemical properties and/or to improve the resolving power. In gradient mode, efficiency cannot be considered, so peak capacity must be used to evaluate performance [99]. Peak capacity describes the number of peaks that can be separated with a resolution of unity during a definite gradient time, and depends mainly on the isocratic efficiency, column dead time, and gradient time. Two properties were selected to evaluate the throughput and resolving power in gradient mode: the gradient time required to attain a peak capacity of 100 (t_{100}) and the maximum peak capacity for a gradient time of 3 h ($P_{3\ h}$).

In two recent papers [90, 100] we applied the strategy developed by Schoenmakers et al. [101] to construct kinetic plots in gradient mode. In this review, a similar approach was employed, using data previously obtained in isocratic mode (H, u, and K_{v0}) for each analytical strategy. Both the lowest gradient times, t_{100} and highest peak capacities, $P_{3\ h}$ which are graphically reported in Fig. 3b, correspond to a pressure drop equal to ΔP_{max} (from Table 2) and consequently to different column lengths and mobile phase flow rates.

Figure 3b presents the data obtained in gradient mode for the model compound with the lowest molecular weight. In terms of throughput, the ranking was similar for the isocratic and gradient modes, and columns packed with small particles are clearly advantageous (i.e. sub-2 μm, UHPLC and HT-UHPLC). It is theoretically possible to attain a P=100 in only 0.5 min with HT-UHPLC, whereas 7 min were required in conventional HPLC. Columns packed with superficially porous sub-3 μm particles (fused-core technology) performed almost equivalently to columns packed with porous sub-2 μm particles (t_{grad} of 1.4 min for P=100), but monoliths and HTLC were less powerful (t_{grad} around 2.5 min for P=100). These theoretical values were confirmed with examples from the literature. For the separation of various pharmaceutical compounds, 50-mm columns packed with porous sub-2 μm or superficially porous sub-3 μm particles produce experimental P values higher than 70 in less than 2 min [102]. Figure 4b shows the efficiency of HT-UHPLC, with a gradient separation of various steroids in less than 45 s, at a temperature of 90°C. Heinisch et al. [24, 103] also showed an impressive chromatogram of nine small aromatic compounds separated in less than 15 s by HT-UHPLC. This separation was performed with a 7.8-s gradient, using a 50×2.1 mm, 1.7-μm column at 2 mL min^{-1} and 90°C.

In terms of the resolving power, monoliths, fused-core, UHPLC, HT-UHPLC, and HTLC offer almost similar peak capacities, ranging between 415 and 480 for a gradient time of 3 h. Compared with isocratic mode, UHPLC and HT-UHPLC generate higher-resolution separations in gradient mode. However, peak capacity is not strictly related to the chromatographic efficiency but also to the column dead time. Because the latter is strongly reduced in UHPLC and HT-UHPLC compared with traditional approaches, the maximum peak capacity for a 3-h gradient is substantially enhanced. Conventional HPLC at 400 and 1000 bar offers 30% less peak capacity compared to the other approaches, demonstrating that columns packed with 5-μm particles do not have any practical benefit in gradient mode. Additionally, sub-2 μm strategies, which

have a maximum pressure of only 400 bar, are also significantly less practical, because of the low permeability of the packing and small backpressure limitation that reduces the column length and mobile phase flow rate that can be used.

In conclusion, the performance obtained in the isocratic and gradient modes is similar, except for UHPLC and HT-UHPLC, which are far more attractive for high-resolution separations in gradient than isocratic mode. The fused-core and UHPLC technologies are very attractive for maximizing both throughput and resolution in gradient mode. These conclusions are in agreement with other studies [7, 23, 97, 98, 102]. Whatever the strategy selected, use of high temperatures is an additional means of improving gradient performance.

Effect of analyte molecular weight on performance

Example of isocratic mode separation

When the molecular weight of the compound is increased to 600 $g mol^{-1}$, the $t_{10,000}$ is 1.5 to 2.5-fold longer (Fig. 5a); it is 2.5 to 6-fold longer for the 1300 $g mol^{-1}$ peptide (Fig. 7a). This behaviour can be attributed to the lower diffusion coefficients D_m of large molecules and thus their lower optimum linear velocities u_{opt} than those of small compounds. In general, high-throughput separations are performed with short columns operating at elevated flow rates, in the C term-dominated region of the Van Deemter curves. However, because the C term is inversely proportional to D_m, longer columns should be employed with

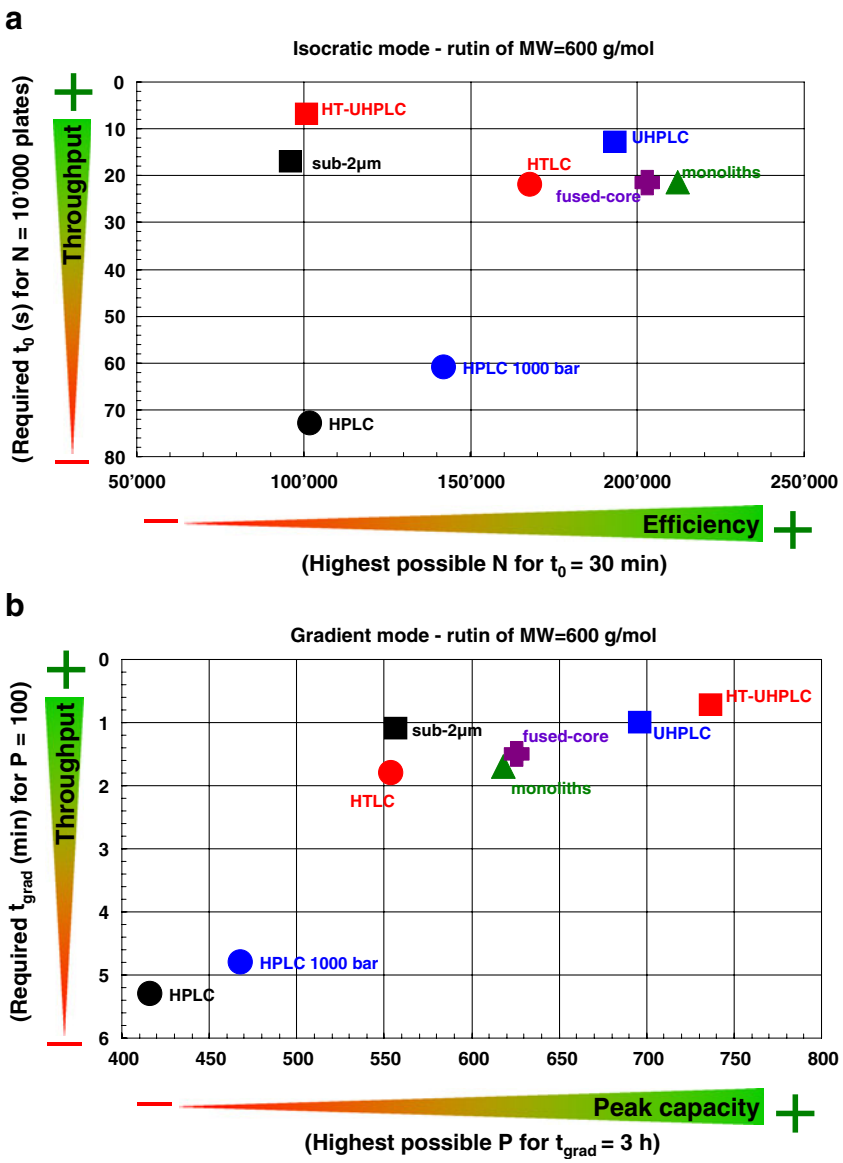

Fig. 5 Performance comparison of LC strategies in terms of throughput and maximum resolution for a model compound, rutin, with MW of 600 $g mol^{-1}$. Similar conditions to Fig. 3

large molecules to compensate for the loss in efficiency resulting from reduced mass transfer, in order to achieve an efficiency of 10,000 plates. The $t_{10,000}$ always increases with the MW of the compound.

On the other hand, because of the lower optimum linear velocities and D_m of high MW compounds, the maximum achievable efficiency for a t_0 value of 30 min is increased by an average of about 1.5-fold with rutin (600 $g mol^{-1}$) and triptorelin (1300 $g mol^{-1}$). To achieve maximum efficiency ($N_{30\ min}$), a long column operating at a low flow rate and in the *B*-term region of the Van Deemter curve should be employed. Because the *B* term is directly proportional to D_m and thus decreases with MW, higher maximum efficiencies ($N_{30\ min}$) can be expected with large molecules.

Despite the increased analysis time for large molecules, UHPLC and HT-UHPLC remain the techniques of choice to maximize throughput for all analytes (Fig. 5a). In terms of maximum achievable efficiency, UHPLC becomes competitive with HTLC, fused-core, and monoliths for larger molecules because of their lower optimum linear velocities. These observations were confirmed by a recent study on the effect of analyte properties on kinetic performance [104]. The authors used UHPLC for compounds with MWs ranging between 200 and 600 $g mol^{-1}$, and concluded that columns packed with small particles require higher efficiencies and lower linear velocities with larger compounds. A significant improvement in high-efficiency values was reported, but an equally important decrease in performance for fast (low-efficiency) analyses was described. Figure 6 presents some separations from the literature of various pharmaceutical substances, with MWs ranging between 266 and 674 $g mol^{-1}$ [105]. With a temperature of 30 °C, separations carried out on a 40-cm column packed with sub-2 μm particles at a pressure of 960 bar and on a 60-cm column packed with superficially porous sub-3 μm particles at a pressure of 621 bar were almost identical, as shown in Fig. 5a. In both cases, N_{max} was around 100,000 plates for an analysis time between 45 and 65 min.

In terms of kinetic performance, there were benefits from increasing the mobile phase temperature up to 90 °C for peptide analysis (Fig. 7a). The best strategies for throughput and resolving power for such biomolecules were HTLC and HT-UHPLC, an observation reported more than 10 years ago by Horvath et al. for separation of peptides and proteins [106–108]. High temperatures can lead to fast and efficient separations of macromolecules with low diffusion coefficients and slow sorption kinetics. These theoretical results suggested that high temperatures could

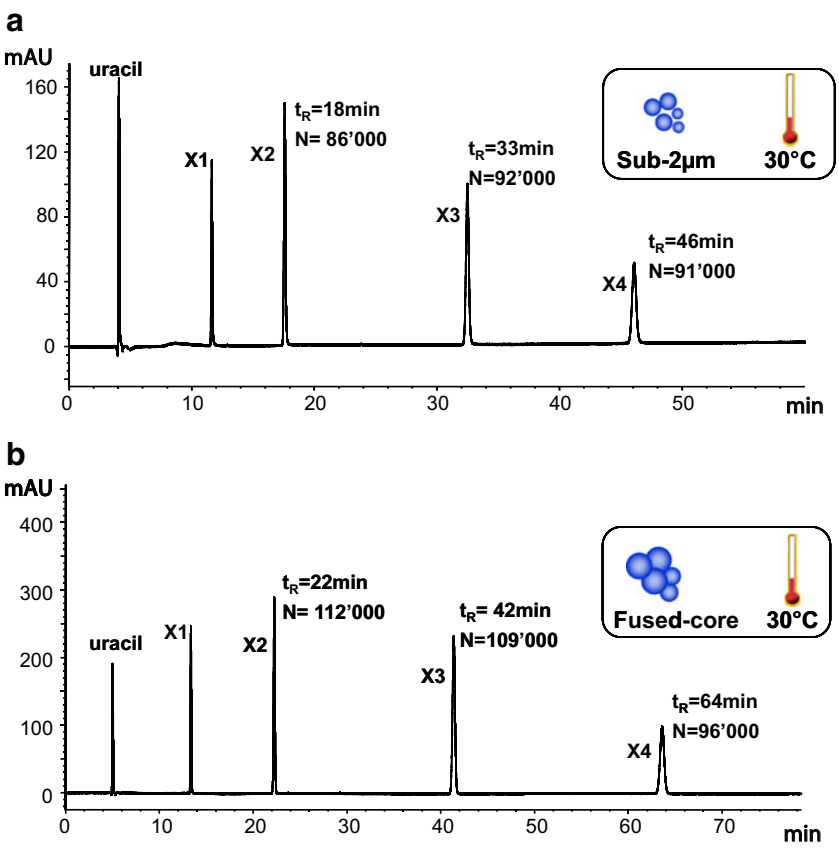

Fig. 6 Highly efficient separations with UHPLC and fused-core technologies. (**a**) chromatogram obtained on four coupled columns at their maximum pressure: a 40 cm Acquity column operated at a flow rate of 0.2 mL min^{-1} and a pressure of 960 bar at 30 °C (mobile phase, 59:41 (*v*/*v*) ACN-H_2O containing 0.1% HCOOH); (**b**) chromatogram obtained on four coupled columns at their maximum pressure: a 60 cm Halo column operated at a flow rate of 0.2 mL min^{-1} and a pressure of 621 bar at 30 °C (mobile phase, 60:40 (*v*/*v*) ACN-H_2O containing 0.1% HCOOH). The retention times and plate counts of each compound are indicated on the chromatograms. Adapted from ref. [105], with permission

generate significant improvement in efficiency, speed and sensitivity [24]. These effects were attributed to:

1. the increase in optimum linear velocity with temperature;
2. the reduction of mass transfer resistance at elevated temperatures; and
3. the modification of secondary interaction kinetics at 90 °C, leading to less broadening and thus higher efficiency.

Surprisingly, there has not been much interest in using monoliths for peptide separation. The performance in terms of both throughput and maximum efficiency is close to that in HPLC. For such analysis, organic monoliths available from several providers would probably perform better than silica-based ones [15, 16].

Example of gradient mode separation

When increasing the molecular weight of the model compound (Figs. 5b and 7b), the gradient time required to attain a P value of 100 remains almost constant, mostly because the rules in gradient mode are different from those in isocratic mode. To obtain the best efficiency in isocratic mode, it is recommended to work close to the optimum linear velocity. In contrast, it has been demonstrated [90, 109, 110] and confirmed in this work, that a very high flow rate in gradient mode is beneficial to the separation quality. The value of P is proportional to the square root of the isocratic efficiency (slightly lower at elevated flow rate, according to Van Deemter), but P also depends on the column dead time, which is inversely proportional to the mobile phase flow rate and seems to be more relevant in

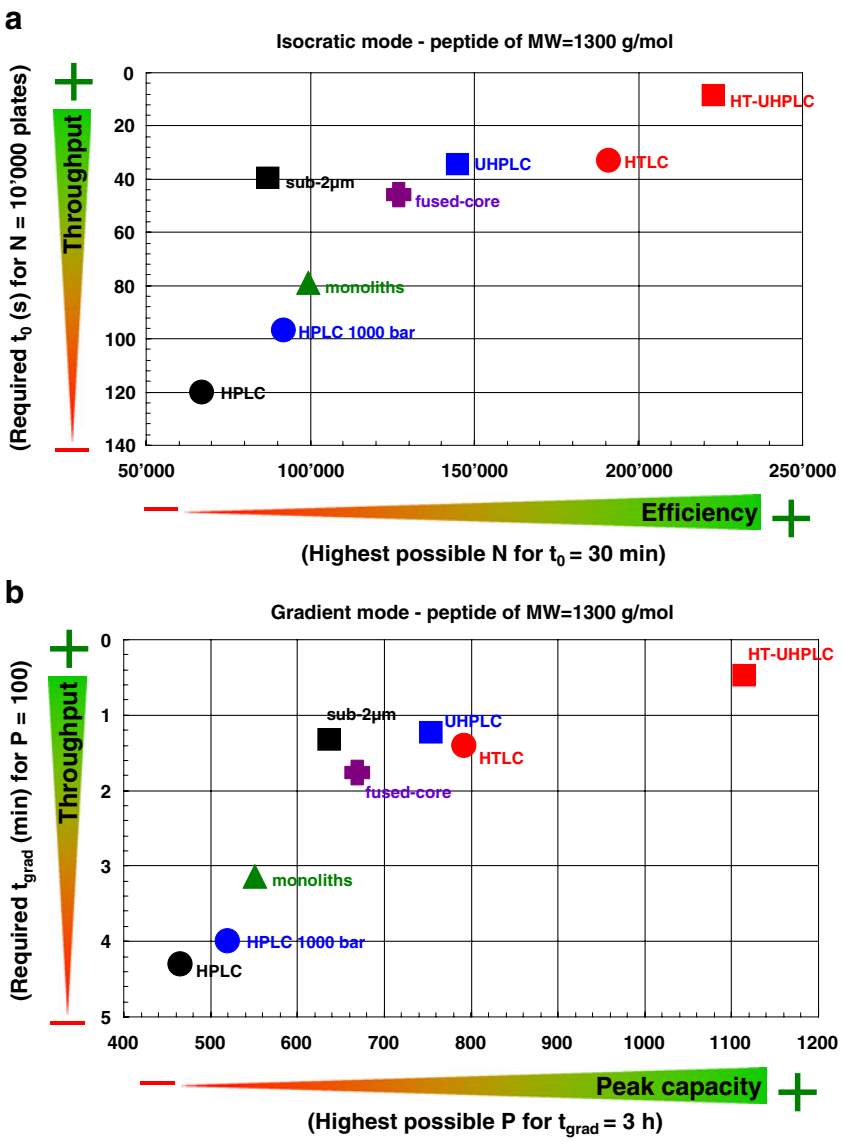

Fig. 7 Comparison of the performance of LC strategies in terms of throughput and maximum resolution for a model compound, triptorelin with a MW of 1300 $gmol^{-1}$. Similar conditions to Fig. 3

the peak capacity equation. In addition, the maximum peak capacity that can be achieved for a gradient time of 3 h increases with the size of the analyte, on average by about 50% and 75% for rutin and triptorelin, respectively. The most impressive increase in P was observed for HT-UHPLC, which increased by 2.5-fold (P=460 for butylparaben vs. P=1110 for triptorelin). The huge improvement is because the optimum column length must be quite long for such gradient times, and, therefore, the mobile phase flow rate is more limited. Longer columns operate at a flow rate closer to the optimum value because of the reduction of diffusion coefficients with the size of compounds. However, HT-UHPLC has not yet been applied for peptide analysis or peptide mapping because of the risk of thermal degradation.

The benefits of HT-UHPLC have been experimentally demonstrated for the metabolite profiling of complex plant extracts (MW around 600 $g mol^{-1}$) by Wolfender et al. [111]. In this study, conventional HPLC was compared with UHPLC and HT-UHPLC. The application of a generic gradient at high temperature was beneficial for extracts containing compounds with a wide polarity range. Indeed, the very high peak capacity obtained at room temperature on long columns in UHPLC was maintained at higher temperatures, and the analysis time was reduced by a factor of two. Furthermore, the authors did not observe degradation of metabolites at 90 °C with a gradient time of 120 min. The combination of UHPLC and HTLC was also applied by Plumb et al. for the profiling of complex biological samples (rat urine) [112]. A very high-resolution separation (P in excess of 1,000) within an acceptable analysis time (1 h) was reported for a reversed-phase gradient at 90 °C.

Conclusion

This work presents an overall comparison of kinetic performance for a variety of analytical systems and analytes. The UHPLC strategy seems to be an attractive technique to speed up or increase resolving power in LC, particularly when used in conjunction with elevated temperatures (HT-UHPLC). However, the best LC set-up should not be selected solely on the basis of kinetic performance, but also taking into account additional features, for example a dedicated chromatographic system, the availability of various chemistries and providers, the column lifetime under extreme conditions, the risk of thermal degradation and the possibility of easy transfer of existing HPLC methods. As such, the HT-UHPLC strategy can appears more critical than the other ones, as it combines small particles, ultra high pressure and elevated temperature and thus, its real interest should be critically investigated. Additionally, it is also important to consider the required column length and mobile phase flow rate of different methods, which can also be regarded as relevant constraints.

The use of columns packed with sub-3 μm superficially porous particles (fused-core) can be a good alternative to UHPLC or HT-UHPLC. Indeed, the fused-core chromatographic performance is very good and certainly could outperform HT-UHPLC with a new generation of columns that would withstand elevated temperatures (up to 90 °C) and higher backpressures (1,000 bar).

On-line 2D LC has become of great interest recently, because 1D LC has a limiting resolving power for very complex samples [113–116]. The data presented in this work can also be used to select an appropriate technique for the first (elevated resolving power) and second (ultra fast separations) dimensions for 2D LC.

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