Kinetic Performance Potential of Different Column Formats and Separation Modes for Liquid Chromatography

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Declaration

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    Causon, T.J., Cortes, H.J., Shellie, R.A., Hilder, E.F.
Abstract

This thesis deals with theoretical and experimental studies on optimisation of separation systems performed using liquid chromatography. The two major themes of work were improving the quality of separations by maximising efficiency with respect to the analysis time (kinetic performance) and by using temperature as an experimental variable. These themes were explored for a range of separation modes and column formats utilised in liquid chromatography.

Included in this thesis is a review of the principles and practices of kinetic performance optimisation for liquid chromatography. Detailed concepts covered in this thesis are introduced and future directions for chromatographic supports discussed.

The role of elevated temperatures (up to 160°C) for practical and performance benefits for organic polymer monolithic stationary phases is examined for typical biomolecule applications. Temperature variation, particularly rapid temperature pulsing, during the separation process is examined using ion-exchange and hydrophilic interaction liquid chromatography modes with capillary columns and a resistively heated column module.

The current performance limitations for the analysis of small anions by ion-exchange chromatography are explored. Additional in silico data are used to transform experimental isocratic data to gradient performance predictions for any normalised ramp rate and physicochemical conditions. Examples of high efficiency (25,000-40,000 theoretical plates) and sample peak capacity ($n_c = 84$) were realised and closely matched predicted values.

A theoretical study of the open tubular column format for liquid chromatography with a thick, porous layer of stationary phase shows the potential of this column format to
improve chromatographic performance with a trade-off for mass loadability. It can be shown that total column diameters should be less than 6 µm with column lengths typically greater than 0.8 m for $N$ values in the range of 125,000–500,000 at a maximum pressure of 400 bar. Elevated temperature LC (90°C) is also shown to increase the allowable total column diameter to up to 9 µm for a larger range of $N$ values (100,000–880,000).
Introduction

1. Background

The term ‘chromatography’ is used to describe a broad collective of techniques applied to the separation of mixtures. In classical chromatography two primary phases exist: a flowing mobile phase for transportation through an immobilised stationary phase support. A mixture of compounds may be introduced to the flowing mobile phase and individual compounds can experience differential migration in this system due to differences in their affinity for each phase (i.e. according to their partition coefficients) leading to separation. Chromatography is a particularly valuable technique as it allows exploitation of the chemical properties of compounds to effect a separation for preparative or analytical purposes.

Currently, the ability of many chromatographic techniques to provide adequate separation is exceeded by the complexity of samples requiring analysis. As the fundamental processes generally remain the same for the wide range of chromatographic techniques that exist, the quality (or performance) of any chromatographic analysis can be subject to broad optimisation strategies based on theoretical knowledge and experiment.

2. Fundamentals of liquid chromatography

2.1. Liquid-solid chromatography

The cornerstone of chromatography is the bulk differences in migration experienced by compounds resulting in separation [1]. While the dynamics of this process at a molecular level are chaotic as each molecule interacts with the stationary and mobile phases independently of the others, compounds are observed at a macroscopic level to migrate smoothly as zones at velocities less than or equal to the bulk mobile phase velocity ($u_0$).
This differential migration exhibited by compounds is determined by the partitioning ratios between the mobile and stationary phases. A retention time \((t_R)\) is recorded and \(u_0\) determined by the residence time of an unretained marker \((t_0)\) with respect to the length of the stationary phase bed. This is frequently quantified for a given compound as the retention factor \((k)\):

\[
k = \frac{t_R - t_0}{t_0}
\]  

(1)

In the case of liquid-solid chromatography, the liquid mobile phase passes through the solid stationary phase; this is commonly shortened to liquid chromatography (LC). The dynamics of LC are somewhat different than for gas phase separations (gas chromatography) as the diffusion coefficients and compressibility of the mobile phase are substantially lower while the viscosity of this phase is much greater. In the case of high pressure liquid chromatography (HPLC), movement of the mobile phase is produced via pressurised pumping of the mobile phase through the stationary phase bed. The stationary phase for HPLC is typically a packed bed of small spherical particles (1-100 μm in diameter) or some form of continuous, permeable structure. Compounds (solute) are dissolved in a liquid medium and introduced as a narrow band into the flowing stream of mobile phase pumped through the stationary phase bed typically contained in a cylindrical column. Solutes that migrate (elute) from the column are collected and/or detected by an analytical device to allow identification and/or quantification of these zones (peaks).

2.2. High pressure liquid chromatography in practice

The principal requirements of any HPLC system are a pump for delivering mobile phase at a constant volumetric flow rate or pressure, an injector for sample introduction, a column
containing a bed of stationary phase and a detector for quantification and/or identification of eluted solutes recorded as a chromatogram.

Pumps used for HPLC must be capable of delivering accurate and reproducible volumetric flow rates with minimal pulsations. The pumping pressures utilised for HPLC are typically less than 400 bar, but recent development in column technology including sub-2 μm particles has necessitated the development of very high pressure pumps capable of exceeding 1000 bar to generate sufficiently high mobile phase velocities. It is also of note that the majority of HPLC pumps are designed to operate in constant flow mode, but it is also possible to operate under conditions of constant pressure. Many HPLC pumps are also capable of mixing and accurately proportioning two or more sources of eluent for the purpose of varying the mobile phase composition during an analysis (i.e. gradient elution).

The injector for HPLC is simply a device employed to accurately introduce a known volume of sample into the moving stream of mobile phase close to the head of the column. Most common instruments utilise two-position valves to divert flow of the mobile phase through a loop containing a set volume of sample.

Columns used for HPLC are highly varied in their length, diameter, type of stationary phase material and chemical functionality. In general the column used must be compatible with the mobile phase employed, have some affinity for the solutes of interest and be sufficiently robust to survive operation under the physicochemical conditions employed. A more detailed discussion of column design is contained in Section 4.

Finally, a detector is required for the analytical purpose of chromatography – that is to quantify and (if possible) identify the solutes in the sample mixture. Detectors may be based on a wide range of detection principles including differences in refractive indices, spectroscopic properties (e.g. ultraviolet wavelength absorbance, fluorescence), aerosol
based (e.g. evaporative light scattering, mass spectrometry) or utilising a post-column reaction of eluted compounds (e.g. electrochemical, chemiluminescence).

3. Performance characterisation and optimisation

3.1. Theoretical plate concept

Eluted solutes detected post-separation typically yield a peak exhibiting an approximately normal (Gaussian) distribution with a standard deviation that is governed by band broadening processes. A convenient method to quantify the amount of band broadening can be found in plate theory, which has its roots in describing distillation processes [2]. While the theoretical validity of this approach is not in agreement with the molecular dynamics taking place, it provides a convenient and accurate method to yield a numerical value for one quality of the separation [1].

In the case of a single chromatographic peak, a relationship between the width of the peak \(w\) and the retention time \(t_R\) describes the “efficiency” of the peak. The value of the theoretical plate number \(N\) can be determined using the retention time at the peak maximum \(t_R\) and the standard deviation of the population \(\sigma\). The standard deviation is estimated by assuming a normal (Gaussian) distribution of the population of solute molecules and can be calculated using Eq. 2 with the corresponding peak width values shown in Figure 1.

\[
N = \left(\frac{t_R}{\sigma}\right)^2 = m \left(\frac{t_R}{w}\right)^2 = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2 = 16 \left(\frac{t_R}{w_{4\alpha}}\right)^2
\] (2)
As separations are performed on columns of a fixed length, the theoretical plate number $(N)$ and the column length $(L)$ are related through the height equivalent to one theoretical plate $(H=L/N)$. The fundamental work of van Deemter [3] provides the framework for describing sources of band broadening during the chromatographic process. The relationship between $H$ and mobile-phase velocity $(u_0)$ is described as the sum of different band-broadening contributions:

$$H = A(d_p) + \frac{B}{u_0} + C(d_p^2)u_0$$

(3)

where the $A$-term is the eddy-diffusion term describing dispersion due to differences in magnitude and direction of the flow through a chromatographic column. The $B$-term describes the longitudinal-diffusion contribution to total band broadening due to Brownian motion. The $C$-term represents the lack of mass equilibrium in the mobile phase $(C_m)$ and that in the stationary phase $(C_s)$.
Clearly, the $A$ and $C$ terms in Eq. 3 can be improved by reducing the particle diameter yielding a lower $H$ value at a given linear velocity. This is illustrated in Figure 2, where plate height values for some typically used particle diameters ($d_p = 2, 3$ and $5 \, \mu m$) are shown for a range of linear velocities. This type of representation is often described as a “van Deemter plot”. It is further possible to define analogous reduced terms (i.e. dimensionless parameters) to allow comparison of columns packed with different particle sizes or column diameters [1].

This type of analysis provides the basis for optimising the theoretical plate number by selection of the optimum mobile phase velocity (i.e. the minimum of the $H, u_0$ curve). However, to compare performance with some consideration to the available time for analysis (i.e. plates/time) the maximum available pressure drop ($\Delta P_{\text{max}}$), the column length and the physicochemical conditions must also be taken into account.

Figure 2. $H, u_0$ curves calculated using Eq. 3 for typical parameters ($A = 1$, $B = 2$, $C = 0.1$) and $d_p$ values of 2, 3 and 5 $\mu m$. 
3.2. Kinetic performance
One possibility for performance characterisation not considered by general plate height equations is the increase in pressure drop with the inverse square of the particle size which limits the maximum column length or mobile phase velocity that can be employed; this affects the maximum plate number that can be generated or the minimum required analysis time.

To address this trade off between \( N \) and time, a graphical approach to compare the kinetic performance in terms of efficiency and analysis time of separations that differ in nature (\textit{i.e.} HPLC versus gas chromatography) was introduced by Giddings in 1965 \cite{4}. This type of plot provides an indication of the time required to obtain a specific number of plates at a given maximum pressure drop for columns of different length. This work was continued by Poppe who introduced a method using iterative calculations to generate a plot of the pressure-drop limited plate number as a function of \( N/t_0 \) \cite{5}. More recently, Desmet \textit{et al.} \cite{6} introduced a series of kinetic plots derived from experimental \( H, u_0 \) and column permeability data for unbiased comparison of different stationary phase formats and chromatographic conditions. A detailed explanation of this type of performance characterisation is given in Chapter I.

3.3. Chromatographic resolution
Beyond optimisation of the plate height for the analysis of a single solute, assessment of the quality of any chromatographic analysis requires a quantitative measure of how well two or more peaks of interest are separated. Plainly speaking, for any two peaks of interest we can consider the width of the peaks, their retention times and the ratio of their respective retention factors (\( \alpha \)). The overall quality of a separation between two peaks is conventionally calculated using the resolution factor (\( R_s \)). Eq. 4 shows that resolution is
influenced by three important factors: the theoretical plate number \((N)\), the retention factor \((k')\), and the selectivity \((\alpha)\).

\[
R_s = \frac{2(t_{R1}-t_{R2})}{w_1+w_2} = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha-1}{\alpha+1}
\]  

Typically the selectivity (mobile and stationary phase chemistry) is optimised prior to the theoretical plate number and kinetic performance. This involves selection of a mobile and stationary phase combination with suitable properties to effect retention and elution of the compounds of interest. After these optimisations, there also remain possibilities for improving the resolution by variation of the mobile phase during the analysis by direct means \((e.g. \ gradient \ HPLC)\) or by indirect means \((e.g. \ temperature \ variation)\). However, these are not exclusive effects and experimental investigation often yields more complex results than predicted by theory \([7]\). Thus there is a wide range of possibilities for optimising both \(N\) and \(R_s\) in chromatographic method development.

### 3.4. Influence of temperature

One variable that can be used to manipulate chromatographic resolution by changing efficiency and retention characteristics is the temperature at which the analysis is performed. Thermodynamically, the retention of components in HPLC can be described by a form of the van’t Hoff equation:

\[
\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \beta
\]  

where \(T\) is the temperature, \(R\) is the universal gas constant, \(\Delta H\) and \(\Delta S\) are the partial molar change in enthalpy and entropy (respectively) of transfer from the mobile to
stationary phase, and \( \beta \) is the phase ratio (i.e. the ratio of volume of mobile phase to that of stationary phase).

From this equation, it can be seen that increasing temperature should result in a predictable decrease in retention. However, deviations from linearity derived from particular functional groups within a solute (e.g. ionisable groups), temperature related changes in the mobile phase (e.g. pH) \([8]\) or variation of the phase ratio \([9]\) may all occur. These selectivity changes can be manipulated to optimise resolution (Eq. 4), which can be a more convenient approach than changing the mobile phase or column.

Increasing temperature should also have a positive benefit on \( H \) values at high linear velocities due to increased diffusion and improved mass transfer. Conversely, the \( B \)-term contribution to band broadening will be significantly increased due to larger diffusion coefficients; this latter effect depends on the molecular weight of the analyte and affects the performance if operating around the optimum of the curve and particularly at sub-optimum linear velocities. These effects are made apparent in the profile of \( H, u_0 \) plots as the influence of the \( C \) term is substantially reduced while the minimum of the curve is shifted to a higher linear velocity value. Furthermore, increased temperature results in a reduction in the viscosity of the mobile phase allowing a wider range of flow rates and column lengths to be employed for improved kinetic performance.

Other than benefits to kinetic performance, resolution can also be controlled by temperature induced changes in selectivity. Temperature variation can also be used analogously to variation of the mobile phase composition during the separation process (i.e. gradient elution) to extend the range of solutes amenable to separation in a single
analysis [10-11]. This can be advantageous in comparison to conventional gradient elution using capillary columns as it eliminates the delay volume problem [12].

4. Column chemistry and morphology

4.1. Mechanisms of interaction

To effect a chromatographic separation, the solutes of interest must exhibit some affinity for both the stationary and mobile phase environments. There is a wide range of chemical properties that can be exploited for this purpose including hydrophobicity, ionic charge, molecular size, entropic effects and chirality. Two of the most commonly employed mechanisms for HPLC are reversed-phase and ion-exchange techniques.

The term reversed-phase was derived to contrast from the terminology applied to early HPLC methods which used unmodified silica or alumina stationary phases with a strong affinity for polar compounds (termed normal-phase). In reversed-phase HPLC retention is dependent upon the hydrophobic properties of compounds and the mobile phase is typically an aqueous-organic mixture (e.g. water-methanol) with a hydrophobic stationary phase (e.g. octadecylsilane). As this environment promotes partitioning of the solute based on its hydrophobicity, reversed-phase HPLC is applicable to a wide range of small to large compounds with low polarity. Reversed-phase columns are the most commonly used for HPLC applications from small molecules to large biomolecules such as intact proteins.

Coulombic interactions provide a convenient method for separation of compounds according to their charge using the principles of ion-exchange (IEX). For this type of analysis, the stationary phase contains chemically bonded ion-exchange sites with fixed ions and oppositely charged counter ions. For example, for the analysis of anions the stationary phase must contain fixed cationic groups to interact with anionic solutes to
retain them (anion-exchange). While the separation of cations requires a stationary phase containing fixed anionic groups with which cationic solutes can interact (cation-exchange). The mobile phase is comprised of a so-called competing ion which drives the equilibrium such that a single monovalent ion displaces a single monovalent counter ion to maintain electroneutrality [13]. Ion-exchange chromatography is frequently applied for analysis of small inorganic and organic ions and also for protein purification.

4.2. Packed columns

Currently the most common column format for HPLC, packed columns are prepared by tightly packing (normally spherical) particles of stationary phase material in a column with frits in place to hold the packed bed in place (Figure 3a). This type of column has a long history of development in reduction of the particle size, modification of chemical functionality, changing the porous properties and improving the regularity of particles and packing structure [14].

Packed columns can be prepared by a number of packing techniques including dry-packing, high pressure slurry packing and supercritical fluid (CO₂) packing [15]. Generally, for particle diameters of less than 20 μm, the high-pressure slurry technique is most commonly used [16]. This involves preparation of a dispersion of particles in a liquid medium that is filled into a reservoir and a high pressure (100-400 bar) applied to pack the particles into a cylindrical column. Numerous types of materials are used for particulate column packings including silica, polymer, zirconia, graphitic carbon and diamond. One important requirement for any packed column is the use of retaining frits at the termini of the column to hold all of the stationary phase particles in the column. In the case of capillary columns (< 0.5 mm internal diameter) this is commonly prepared by heating a
small amount of silica particles inside the column terminus to produce a porous, fused silica frit.

Packed columns remain the performance benchmark for most modes of HPLC. For example, a state of the art 50 cm long capillary column packed with porous sub-2 μm particles can achieve 200,000 theoretical plates for early eluting compounds operating close to the optimum linear velocity applying 1,600 bar in the reversed-phase mode [17]. This excellent performance improvement over early HPLC columns has been due to the extensive theoretical and practical advances in development of small (<3 μm) spherical particles with a narrow size distribution and porous properties tailored to suit diffusive mass transport [17-18].

4.3. Monolithic columns

The term “monolith” in this context refers to a stationary phase format that completely fills the column volume as a 'single particle' of porous material (Figure 3b). This type of stationary phase is characterised by a macroporous, continuous structure typically made from organic polymers [19] or silica precursors [20]. Monolithic columns for HPLC are typically prepared in situ from a mixture of precursors and solvents.

Figure 3. Schematic representation of column formats: (a) packed, (b) monolithic and (c) open-tubular.
The preparation of silica monoliths utilises a sol-gel transition involving the hydrolysis and polycondensation of alkoxides in an alcohol/water/silica system. An understanding of the two known modes of phase separation in this system (nucleation and growth and spinodal decomposition) allows control of the phase separation onset to yield fine or coarse structures without alteration of the gelling/freezing process. This allows all of the pore characteristics to be controlled by adjustment of the starting composition in an isothermally polymerising system [21]. Typical silica monoliths for HPLC are a silica skeleton with a bimodal pore structure of macropores (~1-3 μm) and mesopores (~0.01 μm) and have been most successfully utilised for small molecule separations [20,22].

Polymeric monoliths are prepared from a mixture of organic monomers, porogenic solvents and an initiator. The polymerisation can be initiated by a number of methods, but is typically performed using heat or irradiation. The pore size can be controlled to a certain extent by variation of the type and concentration of monomers, crosslinking agents and porogenic solvents [23]. As a large number of organic monomer precursors exist, there is a wide range of morphological and chemical properties that can be obtained using this approach.

One of the major goals in development of monolithic columns is the increased permeability with respect to packed columns. Coupled with a suitable pore structure, monolithic columns can be made longer and operated at higher linear velocities than packed columns allowing access to improved kinetic performance. Research on new methods for the optimisation of the morphology and porosity has allowed modern silica monoliths to yield improved kinetic performance over conventional packed columns [24]. However, this is currently only possibly for demanding separations requiring large plate numbers (e.g. $N >50,000$) with long columns ($L >30$ cm) [25].
Improving the chromatographic performance of polymer-based monolithic columns is currently more challenging as efficiency is generally poor despite the oft-cited benefits of convective driven mass transfer. Some recent efforts in improving the performance for separations of small molecules has had some success [26-27], but most applications for polymer monoliths demonstrated are for biomolecule separations performed in gradient elution mode where the structure and chemistry of the polymer limits non-specific adsorption in comparison to silica based materials. Notwithstanding, polymer monoliths offer several practical advantages as chromatographic supports including the wide range of available organic monomers, structural integrity, low flow resistance and stability under extreme pH and temperature conditions [28-30].

4.4. Open-tubular columns

Open-tubular columns are characterised by a film of stationary phase coating the surface of the column wall with a large flow-through region (Figure 3c). Despite their unquestioned dominance in capillary gas chromatography (GC), the practical implementations of open-tubular columns as HPLC columns is known to be hindered by the decreased diffusion in liquids (thus requiring narrower column diameters) and limited mass loadability (thus requiring thicker stationary phase coatings) [5,31].

There are a number of approaches for preparing open-tubular columns described in the literature. Most of these pertain to the use of thin, permanent coatings for application in capillary electrochromatography (CEC) [32]. Conversely, columns prepared for use in pressure driven mode typically have thicker films and are often prepared using a polymerisation step akin to those used for typical monolithic columns [33-34]. In this type of preparation, some control of the layer thickness can be achieved by adjusting the ratio of precursor(s) to solvent(s) with respect to the capillary diameter.
Early practical investigations into thin-film open tubular columns [35-38] and theoretical studies [31,39-42] indicated that improvements over packed bed columns can only be obtained when the total column diameter was approximately two times the particle size or less [31]. As such, very narrow column diameters (1-10 μm i.d.) are required, which poses problems due to limitations of detection and mass loadability. However, increasing the thickness of the film shifts the optimum column diameter to larger and more practical values, reducing some of the problems of column manufacturing. The use of thick-films can thus be used to yield trade-off between chromatographic performance and mass loadability [42].

5. Scope of thesis

This thesis primarily focuses on application of theoretical and practical performance optimisation strategies for a range of HPLC approaches. The work has a strong focus on optimisation of the theoretical plate number and kinetic performance (i.e. efficiency requirements) for a variety of chromatographic modes and applications. A secondary focus of the work was improving chromatographic resolution utilising variation of temperature.

Chapter I focuses on the principles and preparation of kinetic plots to characterise the performance in isocratic and gradient LC. This graphical approach allows the selection of columns (i.e. optimum particle size and column length) and LC conditions (operating pressure and temperature) to generate a specific number of plates or peak capacity in the shortest possible analysis time. Instrument aspects including the influence of extra-column effects (maximum allowable system volume) and thermal operating conditions (oven type) on performance are discussed. In addition, the performance characteristics of porous-shell particle packed columns and monolithic stationary phases are presented and the potential of future column designs is discussed.
In Chapter II, the performance of alternative approaches to conventional acetonitrile gradient methods for reversed-phase liquid chromatographic analysis of intact proteins were investigated using commercial poly(styrene-co-divinylbenzene) monolithic columns. Alternative solvents to acetonitrile (2-propanol and methanol) coupled with elevated temperatures demonstrated complementary approaches to adjusting separation selectivity and reducing organic solvent consumption. Only minor (<5%) decreases in detectable protein recovery occurred between 40-100°C on the timescale of separation (2-5 minutes), while reducing the viscosity of a 2-propanol/water eluent by elevating the separation temperature permitted serial coupling of three columns to increase peak production. Narrow-bore (1 mm i.d.) columns were also found to provide a more suitable avenue to fast, high temperature (up to 140°C) separations.

A more detailed kinetic performance appraisal of organic polymeric monolithic columns also using commercial poly(styrene-co-divinylbenzene) monolithic columns was investigated in Chapter III. Analysis of a protein digest sample at elevated temperatures (≥80°C) indicated no apparent solute degradation, but comparison between low molecular weight solute and peptide separations highlighted the markedly different mass transport processes observed on macroporous monolithic beds and an improved $C$ term at elevated temperature in both instances. The current usefulness of this column format for biomolecule analysis was further studied using a kinetic performance characterisation for the first time to provide direction for column development servicing this application.

In Chapter IV an investigation of the performance of polymer-based particulate columns for ion-exchange separations of small inorganic anions using a kinetic plot characterisation is presented. The influence of solute type, particle size, temperature and maximum pressure drop upon theoretical extrapolations was investigated with data collected using anion-exchange polymeric particulate columns. The quality of extrapolations was found to
depend upon the choice of solute, but could be verified by coupling a series of columns. Separations of small anions yielding 25,000-40,000 theoretical plates using five serially connected columns (9 μm particles) were obtained and yielded deviations of <15% from predictions. While this approach for achieving high efficiencies results in a very long analysis time ($t_0 = 21$ min), separations yielding approximately 10,000 theoretical plates using two serially connected columns ($t_0 < 5$ min) were shown to be more practically useful for isocratic separations when compared to use of a single column operated at optimum linear velocity ($t_0 > 10$ min).

This approach was extended using a novel gradient kinetic plot method for the performance characterisation of gradient separations of small inorganic anions in Chapter V. The method employed requires only information obtained from a series of isocratic column performance measurements and in silico predictions of retention time and peak width under gradient conditions. Results obtained under practically constrained conditions allow some solutions to generation of high peak capacities and rapid peak production for fast analysis to be determined. Using this prediction method, a maximum theoretical peak capacity of 84 could achieve separation of 26 components using a 120 minute gradient ($R_s > 1$). This approach provides a highly convenient tool for development of both mono- and multidimensional LC methodologies as it yields comprehensive understanding of the influence of gradient slope, analysis time, column length and temperature upon kinetically optimised gradient performance.

In Chapter VI, the optimisation of open-tubular column design for LC was investigated using a theoretical approach. To alleviate the low mass loadability and a corresponding poor concentration detectability of open-tubular liquid chromatography (OTLC) columns, much effort has been directed toward increasing the surface area and thickness of coatings. The kinetic optimisation of this column format was revisited by taking all thick-film
effects for porous coatings on retention, column resistance, band broadening and mass loadability into account. Considering the most advantageous case (i.e. where the retentive layer allows for the same high internal diffusion coefficient as conventional porous particles), calculations show the need for the development of coating procedures leading to porous films filling up approximately 50 to 70% of the total column diameter. Some predictions of practical targets for OTLC column development are presented including the use of elevated temperatures to increase the allowable column diameter.

Chapter VII explores the use of dynamic temperature changes on chromatographic resolution for ion-exchange separations of small anions. Thermoelectric heating of capillary columns enables accurate temperature gradient programming and rapid temperature pulses during the separation process. This approach has some advantages over solvent gradient elution methods as the rapid change of temperature can enable improved resolution between two peaks of interest without substantially influencing the rest of the separation.

6. Future work

Additional research that can follow the work in this thesis can be considered in three categories: firstly to improve the chromatographic performance of the organic polymer based stationary phases studied in Chapters III-V; secondly to develop new, thick-film open-tubular columns as guided by the theoretical work from Chapter VI; and finally to develop a better fundamental understanding of temperature changes for manipulating retention and selectivity in HPLC as experimentally shown in Chapters II & VII.

Theoretical direction for improving the performance of monolithic supports is provided from a combination of detailed experiment [43-44], computational fluid dynamics [45-46] and an understanding of kinetic performance characterisation [6]. The overwhelming
conclusion of these studies (and supported by experiment in Chapter III) is that currently available monoliths generally suffer from a broad pore-size distribution and poor bed homogeneity. Simply put, monoliths with a reduced domain size and improved bed homogeneity will benefit most from the enhanced permeability of this column format by virtue of reduced eddy dispersion [46]. Some attention has been paid to polymeric monoliths recently with research on shortened polymerisation times [26], variation of polymerisation conditions [47-48] and secondary hypercrosslinking [27]. While these approaches provide some performance improvement (although none have characterised kinetic performance) these columns still lag far behind silica monoliths and particulate columns given the minimum plate heights ($H_{\text{min}}$) are still >20 μm for retained compounds.

This ostensibly leads to two important research goals: 1) improving the structural homogeneity of polymer monoliths and 2) demonstrating applications where these columns provide a clear advantage over alternative technologies. In the case of the former, the task is challenging as it may require novel polymerisation methods using templates [49-50] or silica-polymer hybrids [51] to improve the structural homogeneity and reduce the contribution of eddy dispersion to band broadening. In order to obtain higher efficiencies with monolithic stationary phase technology, new highly ordered structures may be needed and consequently novel polymerisation methods (which sometimes are found in other fields not related to separation science) are required. In this regard, it is also important to consider that quantification of performance improvement for monolithic columns requires measurement of both the efficiency (via the conventional van Deemter approach) and the bed permeability to allow an unbiased estimate of the optimum column performance [6]. In doing so, this avoids erroneous over-estimation of column performance by use of measures such as plates/m as practical column lengths and analysis times can be properly considered.
The polymer-based ion-exchange stationary phases studied in Chapters IV & V can benefit greatly in terms of kinetic performance by decreasing the particle size. Unlike the polymer monoliths, the regularity of packed beds of polymer particles is more easily optimised with good packing procedures and a narrow particle size distribution. Using smaller particles may in turn require higher backpressures to be employed, but as shown by development of small particles for HPLC using reversed-phase materials significant performance improvements are achievable. One difficulty that may be encountered for this approach is the synthesis of very small polymeric particles with ion-exchange functionality.

The development of new thick-film open tubular columns discussed in Chapter VI is a challenging task, but one that can offer great performance if technical challenges can be overcome. Aiming for 5 μm i.d. columns should be an achievable goal if using sensitive mass spectrometric detection. The major obstacles to realising this possibility are the difficulties in manufacturing 1-2 m long columns reproducibly and the instrumental constraints associated with nanolitre scale volumes and detection limits. Notwithstanding these difficulties, some practical development in this area can be achieved particularly if gradient elution mode is employed.

The stationary phase morphology and chemistry play an important role in the future of high temperature HPLC (HTLC) as the stability of many formats is questionable under high temperature conditions. Polymer monolith columns have been shown to have excellent thermal stability under HTLC conditions in Chapter II and provide an ideal platform for research in this field. From an instrumental perspective, Chapter VII illustrates that practical applications of HTLC continue to show most promise using capillary column formats and novel heating devices with thermoelectric elements [52-53]. Despite the lesser effect of temperature on selectivity and retention compared to solvent
strength, the ability to rapidly vary temperature is complementary to that offered by changing the solvent gradient composition as temperature alteration can be transient or confined to one section of the chromatographic column.

The modelling of heat transfer properties in a low thermal mass HTLC system by Verstraeten et al. [54] illustrates the great potential of narrow bore (<1 mm i.d.) columns in conjunction with relatively fast heating and cooling rates. Thus, future work in this area should also be focused on developing strategies for temperature profile optimisation akin to the separation simulation approaches developed for solvent gradient elution profiles. In this regard, applications for ionisable solutes are particularly amenable to this approach as the retention behaviour is often vastly different between solutes contained in typical samples (e.g. exothermic vs. endothermic) and temperature can play a vital role in resolution optimisation.
7. References


8. Acknowledgements

After three years, I can reflect on this undertaking and acknowledge that it would not have been possible without the support of many others.

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Review Article

Kinetic performance optimisation for liquid chromatography: Principles and practice

This HPLC tutorial focuses on the preparation and use of kinetic plots to characterise the performance in isocratic and gradient LC. This graphical approach allows the selection of columns (i.e. optimum particle size and column length) and LC conditions (operating pressure and temperature) to generate a specific number of plates or peak capacity in the shortest possible analysis time. Instrument aspects including the influence of extra-column effects (maximum allowable system volume) and thermal operating conditions (oven type) on performance are discussed. In addition, the performance characteristics of porous-shell particle-packed columns and monolithic stationary phases are presented and the potential of future column designs is discussed.

Keywords: High temperature / Kinetic plot / Monolith / Poppe plot / Ultra high-pressure liquid chromatography

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1 Introduction

Since the introduction of HPLC, there has been a trend towards faster and more efficient separations. This trend especially gained momentum with the introduction of ultra high-pressure liquid chromatography (UHPLC) instrumentation in combination with columns packed with porous sub-2 μm particles in 2004. Mellors and Jorgenson [1] achieved 200 000 theoretical plates (N) for early eluting compounds using a 50 cm long capillary column packed with 1.5 μm porous particles operating close to the optimum linear velocity applying 1600 bar, whereas 150 000 plates could be obtained applying 4400 bar with a 2.5-fold reduction in analysis time. Furthermore, with present day LC, a wide variety of different phases and column formats (e.g. packed columns, monolithic phases, and pillar-array chips) can be selected, which may be used at ultra high-pressure and/or high-temperature conditions. This range of possibilities makes it difficult to select the optimum separation conditions. This tutorial is written in such a way to help the reader to practically employ performance characterisation methods to select columns and chromatographic conditions to achieve the best trade-off between separation efficiency and analysis time.

The fundamental goal in separation science is to separate (and quantify) analytes from each other requiring a minimum resolution factor (R_s) of 1.5. Equation (1) shows that there are three important factors influencing R_s: the theoretical plate number (N), the retention factor (k), and the selectivity (α).

\[ R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha - 1}{\alpha + 1} \] (1)

Typically, the selectivity (mobile and stationary phase chemistry) is optimised first. This involves performing a number of scouting runs on columns with different chemistries and various mobile-phase compositions. Apart from these changes, α can also be altered by modifying the column temperature and/or the gradient slope [2]. Afterwards, optimisation of efficiency and analysis time can be performed. In this regard, increasing the column length (L) in an ad hoc manner to improve efficiency can be useful, but generally leads to a large increase in the analysis time. Instead, tuning the column length together with the stationary phase morphology (e.g. particle size) can result in a better compromise between N and analysis time.

N and L are related through the height equivalent to one theoretical plate (H = L/N) with the relationship between H and mobile-phase velocity (u_0) described by van Deemter as the sum of different band-broadening contributions [3]:

\[ H = A(d_p) + \frac{B}{u_0} + C(d_p^4)u_0 \] (2)

The A-term is the eddy-diffusion term describing dispersion due to the differences in magnitude and direction of the flow through a chromatographic column. The B-term describes the longitudinal-diffusion contribution to total band broadening due to Brownian motion. The C-term represents the lack of mass equilibrium in the mobile phase (C_m) and that in the stationary phase (C_s).

Equation (2) demonstrates the obvious advantage of using small particles (low d_p) to decrease H. However, the
High temperature liquid chromatography of intact proteins using organic polymer monoliths and alternative solvent systems

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Alternative approaches to conventional acetonitrile gradient methods for reversed-phase liquid chromatographic analysis of intact proteins have been investigated using commercial poly(styrene-co-divinylbenzene) monolithic columns (Dionex ProSpherTM RP-2H and RP-4H). Alternative solvents to acetonitrile (2-propanol and methanol) coupled with elevated temperatures demonstrated complementary approaches to adjusting separation selectivity and reducing organic solvent consumption. Measurements of peak area at increasing isothermal temperature intervals indicated that only minor (<5%) decreases in detectable protein recovery occurred between 40 and 100 °C on the timescale of separation (2–5 min). The reduced viscosity of a 2-propanol/water eluent at elevated temperatures permitted coupling of three columns to increase peak production (peaks/min) by 16.5%. Finally, narrow-bore (1 mm i.d.) columns were found to provide a more suitable avenue to fast, high temperature (up to 140 °C) separations.

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1. Introduction

In most reversed-phase high performance liquid chromatography (HPLC) applications for protein analysis the resolution factor ($R_s$) is improved by changing the solvent gradient slope or employment of an alternative column. However, changes in selectivity derived from alteration of temperature and/or the organic modifier can be a convenient approach to improve resolution [1]. In the case of protein HPLC, if columns capable of withstanding high flow rates and steep gradients are also stable at elevated temperatures, other analytical benefits should also be achievable [2].

Recently, Everley and Croley [1] demonstrated how variation of organic modifier (acetonitrile vs. 2-propanol) and moderate temperature changes (35–65 °C) can be used as complementary approaches to adjust selectivity for protein separations using sub-2 μm particles. A secondary benefit of the alternative solvent approach has since become of greater importance due to the recent worldwide shortage of acetonitrile [3], which has highlighted the need for alternative approaches under changing supply conditions. Despite the advantages of combining solvent and temperature changes for this approach, the nature of sub-2 μm particulate stationary phases currently places limitations on maximum flow rate, solvent type and temperature that can be employed.

An alternative to particle based stationary phase formats for protein HPLC are organic polymer monoliths which offer analytical advantages including high permeability, wide range of available chemistries and rapid mass transfer [4]. We recently demonstrated porous polymer monoliths as a high temperature stable stationary phase (up to 250 °C) for the separation of a range of simple, model solutes using a pure water eluent [5]. These promising results suggested that polymer monoliths could be a suitable support format for demonstrating acceptable protein chromatography using high temperatures (>80 °C) to allow the use of strong, viscous organic modifiers such as 2-propanol, extended column lengths or greater linear velocities for fast separations.

Studies of elevated temperature for liquid chromatography using modern thermally stable chromatographic stationary phases have elucidated many benefits of the high temperature liquid chromatography (HTLC) approach [6,7]. These include gains in analysis speed and reduced consumption of organic solvents [8]. Despite the improvements that elevated temperatures can bring to HPLC methodologies, the major restriction in the development of this approach has been the vulnerability of the column [9]. This was highlighted for protein HPLC in Horváth and co-workers seminal work [2,10,11] which predicted that fast, high temperature analysis of biomolecules could become routine once limitations of stationary phase stability could be overcome. The introduction of stERIC protected silica columns has enabled analysis of proteins to be performed at 120 °C, but this approach requires ultrafast separations (<1 min) to prevent analyte degradation on the timescale of the chromatographic separation [12].

Our own studies on the temperature stability of poly(divinylbenzene) monoliths have indicated the excellent thermal stability and ruggedness of this column format at tem-
Kinetic performance appraisal of poly(styrene-co-divinylbenzene) monolithic high-performance liquid chromatography columns for biomolecule analysis

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A B S T R A C T
A broad appraisal of the kinetic performance of organic polymeric monolithic columns is reported using commercially available poly(styrene-co-divinylbenzene) monolithic columns (Dionex ProSwift™ RP-1S). Analysis of a protein digest sample at elevated temperatures (≥80 °C) indicated no apparent analyte degradation using an inert polymeric stationary phase. Comparison between low molecular weight solute and peptide separations highlighted the markedly different mass transport processes observed on macro-porous monolithic beds and an improved C term at elevated temperature in both instances. The current usefulness of this column format for biomolecule analysis was further studied via employment of a kinetic performance characterisation for the first time to provide direction for column development servicing this application.

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1. Introduction

Of current interest in optimising liquid chromatographic separations is the use of kinetic performance evaluation methods to compare chromatographic conditions and support formats for a given separation problem [1–3]. The approach recently introduced by Desmet et al. [4] permits critical unbiased appraisal of emerging trends for liquid chromatography including the use of very high pressure pumps and sub-2 μm particles [5], monolithic silica columns [6] and high temperature liquid chromatography (HTLC) [7,8] using plate height data from single column experiments. Notwithstanding the utility of this approach, to date it has been limited to performance comparison of packed bed and silica monolithic columns for reversed-phase separations of small organic molecules and some pharmaceuticals [9].

Recent trends in the burgeoning field of biomolecules separations have included the use of long columns with large particles [10], employment of elevated temperatures (up to 70 °C) [11] and alternative stationary phase formats including silica-based [12,13] and organic polymeric monolithic columns [14]. As the unique characteristics of large biomolecules should require different optimum chromatographic conditions, some understanding of current chromatographic trends is required for this application. One well-recognised approach to improve efficiency at elevated flow rates is elevated temperatures [15]. However, an oft-cited limitation of this approach has been the limited temperature stability of many column formats when utilised at elevated temperatures [16,17]. Some approaches used to address this shortcoming have included the introduction of sterically protected silica technology [18], zirconia-based particulate [19] and continuous bed [20] column formats, and the use of polymeric stationary phases in both particulate [21] and monolithic [22,23] formats. While all of the aforementioned approaches provide improved column stability under high temperature regimes, silica and zirconia columns are known to suffer from increased catalytic activity toward solutes when employed for HTLC analysis [24–26]. Conversely, polymer based columns have more recently been shown to promote substantially less solute degradation than other stationary phase types [21,25]. Of particular interest in this group are organic polymeric monolithic columns which have been shown to display greater temperature stability over particulate equivalents [22] and can be employed at temperatures up to 250 °C [23]. We recently exploited the excellent temperature stability and decreased catalytic activity of this column format for high temperature (up to 140 °C) separations of intact proteins using commercially available columns [27]. In this case, the use of temperature as a retention modulator without substantial analyte degradation suggested that this approach could be extended to separations of peptides as this is the more common approach for studying the proteome by reversed-phase liquid chromatography [28]. Of further interest in this study was the potential of the approach to influence kinetic performance with respect to the experimental conditions employed including maximum pressure drop and column length utilised.
Probing the kinetic performance limits for ion chromatography. I. Isocratic conditions for small ions

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ABSTRACT

The first use of the kinetic plot method to characterise the performance of ion-exchange columns for separations of small inorganic anions is reported. The influence of analyte type (mono- and divalent), particle size (5 and 9 μm), temperature (30 and 60 °C) and maximum pressure drop upon theoretical extrapolations was investigated using data collected from anion-exchange polymeric particulate columns. The quality of extrapolations was found to depend upon the choice of analyte, but could be verified by coupling a series of columns to demonstrate some practical solutions for ion chromatography separations requiring relatively high efficiency. Separations of small anions yielding 25–40,000 theoretical plates using five serially connected columns (9 μm particles) were obtained and yielded deviations of <15% from the kinetic plot predictions. While this approach for achieving high efficiencies results in a very long analysis time (t₀ = 21 min), separations yielding approximately 10,000 theoretical plates using two serially connected columns (t₀ < 5 min) were shown to be more practically useful for isocratic separations when compared to use of a single column operated at optimum linear velocity (t₀ > 10 min).

1. Introduction

In the realm of liquid chromatographic techniques, ion chromatography (IC) is generally considered to be a separation technique yielding relatively low separation efficiencies when compared to reversed-phase liquid chromatographic methodologies. This is due partly to limitations in the mechanical strength of small polymeric particles and non-metallic instrumental components such as the column housing and extra-column tubing [1,2]. Despite these limitations, developments over the last 30 years in stationary phase design, including a reduction in particle size from 25 to <10 μm and concomitant increase in ion-exchange capacity, have vastly improved IC performance to the point where typical separations of small anions can yield efficiencies of 5–10,000 theoretical plates in <30 min [1]. While this is adequate performance for most applications requiring separation of <20 components, it does not necessarily reflect the current performance possibilities of the technique.

Approaches to improving chromatographic performance to satisfy analytical goals most commonly revolve around resolution optimisation. In the case of IC, existing approaches to separation prediction using in silico methods with commercially available software (Virtual Column™, Dionex) already serve to minimise time spent on method development by use of solvent strength modelling for selectivity optimisation [3]. However, such an approach cannot be employed for predicting separation performance outside the range of standard column lengths, flow-rates and pressure drop conditions. Such an optimisation requires use of kinetic performance methods first introduced by Giddings [4], developed extensively by Poppe [5] and more recently advanced by Desmet et al. [6].

While the range of IC separation media and instrumentation places some physical limitations on the obtainable efficiency values for this technique, the kinetic performance limits are certainly under-explored. Fortunately for this purpose, an accessible and diverse range of kinetic plots has been introduced recently by Desmet et al. [6] for the unbiased optimisation and comparison of liquid chromatography systems with different support formats under kinetically optimised conditions. Although application of this approach has been limited thus far to a small range of reversed-phase [7–10] and hydrophilic interaction (HILIC) [11] separations of small organic molecules and some pharmaceuticals, there is no theoretical impediment to employing this method for other modes of liquid chromatography, including ion-exchange chromatography.

Predicting any improvements in the kinetic performance for liquid chromatography is best achieved by considering an appropriate number of experimental variables including flow-rate, temperature, column length and pressure drop limitations. Such an approach should allow a reliable appraisal of the current performance limitations of anion-exchange chromatography with...
Probing the kinetic performance limits for ion chromatography. II. Gradient conditions for small ions

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1. Introduction

One mode of liquid chromatography (LC) with a long history of in silico optimisation is ion chromatography (IC) [1–5]. Numerous hard and soft modelling procedures have been described for the analysis of small ionic species allowing development of analyte retention models for computer-based simulations to select optimum conditions for separation. This approach has been developed substantially beyond isocratic modelling to incorporate both single [4] and multistep gradient profiles [6,7], which are commonly employed for the analysis of small ionic species [4] and large ionicogenic pharmaceutical compounds [8]. The primary aim of these simulations is to provide a rapid in silico approach to method development allowing variation in the elution profile to predict optimum separation conditions.

However, this method only allows for the peak capacity to be varied through alteration of the gradient profile. Changes in the morphology of the stationary phase, flow-rate, available pressure drop and column length can also be utilised to improve separations by virtue of increased efficiency and/or speed. There is good reason to understand the influence of these factors for IC given recent approaches for fast separations including the use of short (3 cm) particulate columns [9,10] and highly permeable silica monolithic columns [11–13]. Conversely, the development of high peak capacities for IC has received much less attention in spite of the substantial improvements in column technology [5] and the recent implementation of IC × IC methodologies [14]. The validity of any approach for improving peak production and/or total peak capacity should be assessed using a method which accounts for different support formats and analysis conditions.

Methods for prediction of efficiency under kinetically optimised conditions have been demonstrated to provide practical solutions for method development [15]. The most recent innovation in this area has been the proliferation of graphical kinetic plot (KP) optimisation approaches for quantifying the influences of maximum inlet pressure, particle size, temperature and column length [16]. Most examples of this approach thus far have been confined to the reversed-phase separations of small molecules and pharmaceuticals [17,18], but some more recent examples have employed this approach for the analysis of peptides [19], hydrophilic interaction liquid chromatography (HILIC) [20] and isocratic IC separations [21]. Of these examples, the majority have been tailored for the optimisation of efficiency under isocratic conditions, but the KP method can also be readily extended for understanding the more useful metric of peak capacity under normalised gradient conditions [22].

As IC is particularly dependent upon the implementation of gradient elution profiles to yield satisfactory peak capacities [5], it would seem that separation optimisation procedures should consider the influence of a wider range of analytical conditions upon total peak capacity and peak production. Coupled with a numerical model for prediction of retention time and peak width [6], it
Kinetic optimisation of open-tubular liquid-chromatography capillaries coated with thick porous layers for increased loadability

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The kinetic optimisation of open-tubular liquid chromatography (OTLC) columns has been revisited by taking the thick-film effects for porous coatings on retention, column resistance, band broadening and mass loadability into account. Considering the most advantageous case (i.e. where the retentive layer allows for the same high internal diffusion coefficient as conventional porous particles), calculations show the need for the development of coating procedures leading to porous films filling up approximately 50–70\% of the total column diameter. Furthermore, to achieve optimum kinetic performance for separations of small molecules with total analysis times of less than 8 h (\(k\approx 9\)), total column diameters should be less than 6 \(\mu\)m with lengths typically greater than 0.8 m for \(N\) values of 125,000–500,000 at a pressure of 400 bar. The use of elevated temperature LC (90 °C) is also shown to increase the allowable total column diameter to up to 9 \(\mu\)m for a larger range of \(N\) values (100,000–880,000).

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1. Introduction

Despite their unquestioned dominance in capillary gas chromatography (GC), the practical implementations of open-tubular columns for liquid chromatography columns is known to be hindered by the decreased diffusion in liquids (thus requiring narrower column diameters) and mass loadability restrictions (thus requiring thicker films) \([1,2]\). Significant interest in OTLC column development was apparent in the 1970s and 1980s, but experimental studies on preparing coated capillary columns were almost abandoned during the 1990s primarily due to practical limitations. Development of capillaries with thin-film and etched coatings have continued to be studied particularly for capillary electrochromatography (CEC) \([3,4]\), but these still exhibit low mass loadability and retention capacity.

Early practical investigations into thin-film OTLC columns \([5–8]\) and theoretical studies in the 1970–1990s \([1,9–12]\) indicated that improvements for OTLC over packed bed columns can only be obtained when the total column diameter was approximately two times the particle size or less \([1]\). This led to predictions of very narrow and seemingly impractical column diameters (less than 2 \(\mu\)m i.d.) given the limitations of detection and mass loadability of thin-films. In a later series of papers, Poppe and co-workers \([12–16]\) pointed to the possibility of using thick-films to increase the mass loadability of OTLC columns and alleviate (to some extent) the detection problems. This pursuit of thick-films also shifts the optimum column diameter to larger and more practical values, reducing some of the problems of column manufacturing. Some renewed interest in capillary columns coated with thicker films has been shown recently with practical investigations of OTLC columns for capillary electrochromatography \([17]\) and for analysis of biomolecules by gradient LC \([18–23]\). Although the use of thick-films leads to a trade-off between chromatographic performance and mass loadability all of these studies have favoured columns with relatively thick films in efforts to obtain suitable mass loadability properties for real world applications.

Despite the potential advantages of thick-film OTLC columns, previous theoretical optimisation work has been carried out assuming either a zero \([10]\) or thin-film approximation \([9]\) for the contribution of the resistance to mass transfer in the stationary phase (\(C_S\)) term. However, the thin-film approximation is no longer valid for a film thickness ratio (\(\delta = d_f/d\), Fig. 1A) of greater than 0.1 as the annular geometry of the film starts to play a role thus requiring the full Aris solution to be employed \([24,25]\). Furthermore, to the best of our knowledge, no previous optimisation study has taken the effect of the film thickness on the observed \(t_r\)-based flow resistance (\(\Phi_0\)) into consideration as this has been assumed to remain constant at 32 as the film thickness is increased; an assumption that is again based on a thin-film approximation.

In the present study we therefore revisit the optimisation of OTLC column design considering all of the thick-film effects for truly porous stationary phase films and compare the resulting optimum
Temperature Pulsing for Controlling Chromatographic Resolution in Capillary Liquid Chromatography

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ABSTRACT: In this study we introduce the implementation of rapid temperature pulses for selectivity tuning in capillary liquid chromatography. Short temperature pulses improved resolution in discrete sections of chromatograms, demonstrated for ion-exchange chromatography (IC) and hydrophilic interaction chromatography (HILIC) modes. Using a resistively heated column module capable of accurate and rapid temperature changes, this concept is first illustrated with separations of small anions by IC using a packed capillary column as well as a series of nucleobases and nucleosides by HILIC using a silica monolithic column with zwitterionic functionality (ZIC-HILIC). Both positive (increasing temperature) and negative temperature pulses are demonstrated to produce significant changes in selectivity and are useful approaches for improving resolution between coeluted compounds. The approach was shown to be reproducible over a large number of replicates. Finally, the use of temperature gradients as well as other complex temperature profiles was also examined for both IC and HILIC separations.

The role of temperature in controlling selectivity for liquid chromatography (LC) has been historically viewed as less useful than variation of the eluent strength (e.g., the amount of organic modifier). However, numerous studies using modern instrumentation and stationary phases with improved thermal stability have demonstrated the benefits of elevated temperatures and temperature control for LC.1−4 Although most of these studies have focused on the use of elevated temperatures for isothermal analyses primarily to improve chromatographic efficiency at increased flow rates, temperature variation during the separation (e.g., application of thermal gradients) can also be a useful tool in resolution optimization, particularly where the influence of temperature is strongly complementary to that of solvent strength.5−8

One of the major stumbling blocks in utilizing temperature changes during LC analysis is the limitation of conventional column diameters (i.e., 2.1−4.6 mm i.d.) and the consequent radial temperature gradients.7 Instrumentation equipped with mobile phase preheating does improve this situation, but rapid temperature changes (>10 °C/min) or high flow rates for fast analysis are difficult to employ effectively.9 Consequently, use of capillary LC instrumentation allows many of the advantages of temperature changes to be realized including improved efficiencies at elevated flow rates and control of selectivity in an analogous manner to variation of the mobile phase composition during the separation process, i.e., solvent gradient elution.7 Furthermore, the use of thermal gradients can be advantageous in comparison to implementing conventional solvent gradients with capillary LC instrumentation as it eliminates the delay volume problem and also minimizes the re-equilibration time.10

As has been noted by a number of studies,3,11−15 it is important to recognize that the influence of temperature on selectivity is complementary to that of solvent strength. This is particularly relevant for the LC analysis of ionizable and polarizable solutes as both exothermic and endothermic retention behavior can be observed allowing temperature to play a significant role in selectivity tuning. For example, retention in ion-exchange chromatography (IC) is known to be strongly influenced by temperature16−18 with many anionic solutes exhibiting an increase in retention with elevation of the column temperature indicating an endothermic molar retention enthalpy (ΔHk) according to the van’t Hoff equation:19

\[
\ln k = \frac{\Delta H_R}{RT} + \frac{\Delta S_R}{R} + \ln \phi
\]  

where ΔS_R is the molar retention entropy and ϕ is the phase ratio.

In the case of IC analysis of small anions, the study of Hatsis and Lucy16 classified three groups of retention behavior for some typical small inorganic anions: singly charged, small ions (e.g., nitrate, nitrite) with weakly endothermic or exothermic retention behavior, multiply charged ions with strongly endothermic retention behavior (e.g., sulfate, thiosulfate), and...