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THOMAS THEMELIS
Thesis submitted in fulfilment of the requirements for the award of the degree of
Doctor of Engineering Sciences (Doctor in de ingenieurswetenschappen)

DESIGN OF HIGH-RESOLUTION CHROMATOGRAPHIC APPROACHES:
FROM OPTIMIZATION OF UNIDIMENSIONAL LIQUID CHROMATOGRAPHY TO
ENGINEERING OF MICROFLUIDIC DEVICES FOR SPATIAL MULTI-DIMENSIONAL
SEPARATIONS

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Sample complexity constitutes the major bottleneck in modern life-science research and to tackle this issue powerful separation technology is required. The research performed and reported in this thesis aims in advancing spatial three-dimensional liquid chromatographic (3D-LC) technology as means of efficient and fast separation of complex mixtures.

Current gold-standard proteomic approaches rely on one-dimensional (1D-)LC coupled to tandem mass spectrometry (MS/MS) using long columns, packed with state-of-the-art sub-2 μm particles. Nevertheless, the achieved resolving power is rather limited compared to the complexity of the sample and as a result the detection of analytes via the MS is significantly hampered. Conventional column-based multi-dimensional (MD-) LC techniques, including two-dimensional (2D) and three-dimensional (3D)-LC, offer higher total peak capacity (i.e., a metric of efficiency) but also employ much longer times for the completion of the analysis. This is attributed to the sequential storage of the analyzed fractions eluting out of one dimension and their injection in the following.

An alternative approach to conventional MD-LC is spatial multi-dimensional chromatography. As a concept, spatial separations have existed for many years in different formats i.e., 2D thin-layer chromatography (2D-TLC) and 2D polyacrylamide gel electrophoresis (2D-PAGE). In such cases, analytes are initially separated along the first dimension (1D) development, until the least retained one reaches the end of the strip. Following, all the fractions are simultaneously transferred in the second dimension (2D) and are developed in parallel again until the least retained analyte reaches the end of the separation space. Finally, the compounds are separated in the space domain (X-Y plane) and each band has a distinct pair of coordinates. In the case of spatial 3D-LC a three-dimensional separation body has been envisioned since the first conceptual research attempts. After completing the 1D and 2D developments, a cube is attached at the top of the 2D device, and the third dimension (3D) development is carried out in parallel for all the separated bands distributed in the X-Y plane. As such, that the separated analytes end up having spatial (X-Y-Z) coordinates after the analysis is completed.

The main advantage of spatial multi-dimensional separations lies in the word “parallel” that describes the development of all fractions in the 2D and 3D as opposed to “sequential” used in the conventional MD-LC approaches. This greatly reduces the employed analysis times while maintaining high separation efficiencies, making the technology potentially suitable for screening of a multitude of complex samples.

The scope of the presented doctoral thesis is two-fold. First the assessment of the maximum resolving power in 1D-LC and second to advance the technology involved in the
development of microfluidic devices for spatial MD-LC leading to unmatched separation performance.

In Chapter 2, basic concepts of one- and multi-dimensional separations are introduced and in Chapter 3 the development and optimization of a 1D-LC method with the use of the kinetic performance limits approach for the analysis of a complex mixture of small molecules (MW < 1000 Da) characterized by a broad hydrophobicity range, i.e., antibiotics as a case study, is presented. Chapter 4 provides an introduction to spatial 3D-LC as well as a tutorial approach on the bottlenecks that must be resolved in order to fully develop this technology. Chapter 5 demonstrates possible solutions for one of the major bottlenecks, namely the flow control in microfluidic devices for spatial MD-LC. Chapter 6 discusses the kinetic performance limits of a spatial 3D-LC device integrating a specific combination of separation mechanisms and how certain design aspects like the number and length of the channels in the chip can affect these. Concluding remarks and future prospects are presented in Chapter 7.
CHAPTER 2

INTRODUCTION TO THE BASIC CONCEPTS OF ONE- AND MULTI-DIMENSIONAL SEPARATIONS
2.1. Pressure-driven separations

In pressure-driven separations the generation of the flow that propels the flow through the column, where the separation process takes place, comes from a pump which can operate at a maximum pressure that ranges from 400 bar for conventional high-pressure liquid chromatography (HPLC), up to 1500 bar which is the limit of state-of-the-art, commercially available, ultra-high-pressure liquid chromatography (UHPLC). The linear velocity \( u_0 \) at which the front of the mobile-phase travels in the column is described by Darcy’s law:

\[
    u_0 = \frac{\Delta P \cdot d_p^2}{\phi \cdot \eta \cdot L}
\]

where \( \Delta P \) is the pressure drop along the column, \( d_p \) the particle size, \( \phi \) the flow-resistance factor, \( \eta \) the mobile-phase viscosity, and \( L \) the column length. Through the years there have been developed various type of column chemistries aiming at the separation of analytes with different physicochemical properties. The most important separation modes are summarized in this chapter.

Normal-phase (NP) liquid chromatography was the first separation mode to be used and is carried out on polar stationary-phases based on bare silica or silica modified with cyanopropyl or aminopropyl functionalities. A non-polar/moderately-polar mobile-phase is used typically consisting of \( n \)-hexane mixed with modifiers such as tetrahydrofuran or dichloroethane (amongst others) aiming to adjust the polarity accordingly [1]. The analytes separated with NPLC demonstrate rather low solubility in water and exhibit a polar character, interacting with the stationary-phase via dipole-dipole forces or hydrogen bonding. In 1990 Alpert introduced hydrophilic interaction liquid chromatography (HILIC) as a variation of NPLC where, again, polar stationary phases are employed (e.g., bare silica, diol, zwitterionic) but a mixture of acetonitrile and water are used as mobile-phase [2]. When a hydrophilic chromatography column is eluted with a hydrophobic (mostly organic, >60-70%) mobile-phase, retention increases with the hydrophilicity of solutes, making this mode particularly useful for the separation of proteins, peptides, amino acids, and oligonucleotides [2].

Nowadays, the approach that is used to solve the majority of separation problems (accounts for over 80% of HPLC applications [1]) is reversed-phase liquid chromatography (RPLC). In this mode analytes partition between a non-polar stationary phase (e.g., C₄, C₈, C₁₈) and a polar (mostly aqueous) mobile-phase and are separated based on their hydrophobicity [3,4]. The retention is governed by a combination of intermolecular interactions between the
solute, the mobile-phase and the stationary phase, including dispersion forces resulting from
instantaneous dipole moments and $\pi$-$\pi$ interactions between aromatic or unsaturated molecules
[3,5,6]. As the strength of these interactions increases, the solute is increasingly retained.
Conversely, by increasing the hydrophobicity of the mobile phase the solute’s retention
decreases.

In hydrophobic interaction chromatography (HIC), much like RP analytes (mostly
large biomolecules) are separated based on their differences in hydrophobicity and the used
stationary phase is of moderate hydrophobicity (typically C$_4$ chemistry). The mobile phase
does not contain any organic modifier but instead an inverse salt gradient is applied to elute the
biomacromolecules. At the beginning of the gradient the high ionic strength of the mobile phase
breaks the structured water layers that shield the analytes from interaction with the stationary
phase. This leads to an increase in entropy which is the driving force of the separation. As the
salt concentration gradually decreases, the hydrophobic interactions weaken, leading to the
elution of the biomacromolecules with lower hydrophobic surfaces, followed by those with
high surface hydrophobicity [7].

Ion exchange (IEX) chromatography is a type of LC in which analytes are separated
by adsorbing onto a support containing oppositely charged moieties on its surface followed by
elution using a mobile phase with a gradually increasing ionic strength [8,9]. The higher the
charge density of an analyte the more it will be retained. Depending on the charge of the groups
in the stationary phase, the types of ions that bind to the column may be either cations (i.e.,
positively charged ions) or anions (i.e., negatively charged ions). These two methods are
referred to as cation-exchange with the stationary phase usually made up of sulphonic acid
moieties and anion-exchange chromatography where stationary phase contains usually
quaternary ammonium [8,9].

Size-exclusion chromatography (SEC) separates macromolecules (synthetic or natural
polymers) based on size, or more specifically on Stokes radii [10]. The analytes diffuse in and
out of the accessible by them stagnant pores of the stationary phase and the entire process
occurs within well-defined retention volumes ranging between the interstitial volume of the
column and the sum of the interstitial and intraparticle volume [11]. Typical stationary phases
for SEC of proteins utilize diol modified silica beads while cross-linked styrene is still widely
used for polymer separations in non-aqueous media [12].
2.2. Electro-driven separations

In electro-driven (or electrophoretic) separations the analytes that have a net charge move differentially under the influence of an electric field [13]. The migration of the charged analytes is governed mainly by their size and net charges, and their electrophoretic mobility ($\mu_E$) that can be related to the charge-to-mass ratio is described as:

$$\mu_E = \frac{q}{6\pi \eta r} \quad (2.2)$$

where $q$ is the net charge, $\eta$ the viscosity of the medium and $r$ the radius of the analyte. The actual electrophoretic velocity with which the molecules move is calculated as:

$$v_E = \mu_E \cdot E \quad (2.3)$$

with $E$ being the strength of the electric field.

Capillary electrophoresis (CE) is a high-efficiency separation technique that is performed in narrow bore capillaries (typically 25-100 $\mu$m) filled with buffer solution, without using stabilizing gels [13]. It was first described by Jorgenson et al. in 1983 as a complementary approach to gel electrophoresis and allows the application of higher voltages due to the more efficient heat dissipation coming as a result of using narrow capillaries [14]. This leads to less Joule heating and hence less band-broadening effects, yielding increased efficiencies and significantly reduced analysis times. Being an electrophoretic technique, it follows the same separation principle with the addition of a second type of flow, called electro-osmotic (EOF), which is present when the capillary is filled with a buffer at a pH greater than 3 and the SiOH groups lose a proton to become SiO- ions and is characterized by a flat flow profile. Under this condition the capillary wall is negatively charged and develops a double layer of cations attracted to it. The inner cation layer is fixed, while the outer layer is free to move along the capillary leading to movement of cations towards the cathode under the effect of the electric field which can be mathematically described as follows:

$$\mu_{EOF} = \frac{e\zeta}{\eta} \quad (2.4)$$

where $\epsilon$ is the dielectric constant and $\eta$ the dynamic viscosity of the solution and $\zeta$ the zeta potential [13]. There are several variations of capillary zone electrophoresis that have been widely applied in bioanalysis, including capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) a unique separation mode that combines the use of charged stationary phase and electro-driven flow [13].
Another major type of electro-driven separation is isoelectric focusing (IEF) where charged analytes move differentially based on their isoelectric point (pI), in a medium that forms a pH gradient under the effect of a constant electric field. When they reach the point where the pH equals the pI then the analytes are no longer charged, and their migration stops [15]. In this technique it is important that the sole driving force for the movement of the analytes is electrophoretic mobility, hence the absence of any bulk flow due to EOF is required in order to achieve efficient separations. Carrier ampholytes of different pH ranges have been used to establish the needed pH gradient which can be prepared either in fixed formats called immobilized pH gradients, that use mainly gels (e.g., polyacrylamide, agarose), or even in free solution in case of capillary or microchip IEF (cIEF, mIEF).

### 2.3. Metrics of separation performance in chromatography

During a chromatographic process the injected analytes undergo two different processes which influence the quality of their separation. The first comes as a result of the different affinity that each of them demonstrates for a specific separation mechanism, based on their physicochemical properties, and leads to a proportional increase of the distance between the separated bands as they move along the column. While this differential migration occurs, the bands are also broadening proportionally to the square root of the distance that they have travelled [16]. The combination of these two phenomena leads to a concentration profile of the migrating band that can be ideally described as a Gaussian function and the width of the peak is defined as its second moment that describes the statistical variance, $\sigma^2$ (Fig. 2.1).

![Figure 2.1](image.png)

**Figure 2.1.** Signal response as a function of space or time, showing a Gaussian distribution characterized by its peak width ($W$) and standard deviation ($\sigma$).
According to chromatography theory, a separation process can be considered as a series of consecutive equilibrium steps that the analytes undergo between the mobile and the stationary phase, the number of which is directly proportional to the quality of the separation. Different approaches to measure separation performance have been derived and used.

### 2.3.1. Plate number and plate height

A chromatographic peak is a statistical distribution of molecules and its width is related to the extent of dispersion or band-broadening that occurs during the separation, which is proportional to the square root of the column length \([17]\). This can be described mathematically as follows:

\[
H = \frac{d\sigma^2}{dl}
\]

where \(H\) is the height equivalent of a theoretical plate (HETP) or simply plate height. This is a concept that originates from distillation theory and has been adapted \([17]\). Practically, the lower the height of the theoretical plates, the higher is the number of plates that fit in a column of length \(L\) and hence the higher the efficiency is. This can be formulated as:

\[
N = \frac{L}{H} = \left(\frac{t_R}{\sigma_t}\right)^2
\]

which expresses that the plate number is also equal to the ratio of the time needed by an analyte to elute out of the column \((t_R)\) and its recorded peak-width \((\sigma_t)\).

Van Deemter in his seminal research, plotted the plate height as a function of linear velocity \((u_0)\) deriving a plot (see Fig. 2.2) and a formula that describe dispersion as the sum of three independent terms \([18]\):

\[
H = A + \frac{B}{u_0} + C \cdot u_0
\]

A is the velocity independent eddy-dispersion term (see Fig. 2.2, orange line and insert B) influenced by the particle size \((d_p)\) and packing homogeneity which describes the different path-lengths and velocities that the molecules assume when moving in a column via tortuous paths (bed tortuosity, \(\lambda_{bed}\)) \([20,21]\)

\[
A = \lambda_{bed} \cdot d_p
\]
The B-term (Fig. 2.2, gray line and insert C) represents the molecular diffusion (in the axial direction), the extent of which increases with the residence time of the band inside the column making this process inversely proportional to the linear velocity.

\[ B = 2 \cdot \gamma \cdot D_m \]  \hspace{1cm} (2.9)

It is also directly proportional to the obstruction factor (\(\gamma\)) of the packed bed and the diffusion coefficient of the analytes (\(D_m\), typical values in the range of \(10^{-9}\) m\(^2\)/s for small molecules and \(10^{-11}\) m\(^2\)/s for large molecules) [22].

C is the mass-transfer coefficient that reflects the rate of the equilibrium process between the moving zone and the stationary one (Fig. 2.2, yellow line and insert D). It becomes more difficult to establish equilibrium at faster linear velocities and thus the contribution to band broadening due to mass transfer increases with flow rate. The C-term can be further split in two major contributions; \(C_m\) for the tendency of the molecules to follow different flow-streams with different velocities when moving within the interstitial volume of the column interacting with the surface of the stationary phase, and \(C_s\) that shows how slow/fast the molecules diffuse through the stagnant mobile phase of the pores to the internal surface of the stationary phase [22,23].

\[ C = C_m \left( \frac{k''}{1+k''} \right)^2 \frac{d_f^2}{D_m} + C_s \frac{k''}{(1+k'')^2 D_{pz}} \]  \hspace{1cm} (2.10)

where \(d_f\) is the thickness of the stationary film, \(D_{pz}\) the diffusion coefficient in the porous zone.
of stationary phase and k'' is the zone retention factor which characterizes the equilibrium of the molecules inside and outside the particles and is defined as:

\[ k'' = \frac{t_R-t_i}{t_i} \]  

(2.11)

with \( t_i \) the residence time of an analyte that cannot penetrate the particles. Using HETP allows to compare the efficiency of different columns and Giddings advanced this concept by introducing the reduced plate height (\( h \)), a dimensionless value obtained by dividing \( H \) with \( d_p \) (\( h=H/d_p \)), which when plotted against the reduced velocity (\( v \)) allows to compare experimental data obtained from columns with different characteristic sizes (e.g., particle size) [24]. The reduced plate height can be calculated as:

\[ h = a + \frac{b}{v} + c \cdot v \]  

(2.12)

where \( a, b \) and \( c \) are the reduced (dimensionless) Van Deemter parameters, and the reduced velocity can be described as:

\[ v = \frac{w_d_p}{D_m} \]  

(2.13)

2.3.2. Resolution

A way to assess how well two peaks are separated is by measuring their resolution, i.e., the ratio of their difference in retention times over the sum of their peak widths:

\[ R_s = \frac{t_{R,2}-t_{R,1}}{0.5(w_1+w_2)} \]  

(2.14)

\( R_s \) should be (at the best case) higher than 1.5 to be able to have peaks separated at the baseline and hence be able to properly quantify the analytes if needed. Another equation for resolution was derived, by Purnell [25]

\[ R_s = \left( \frac{\sqrt{N}}{4} \right) \cdot \left( \frac{\alpha-1}{\alpha} \right) \cdot \left( \frac{k_2}{k_1} \right) \]  

(2.15)

expressing it as a function of \( N \), the retention factor (\( k = t_R-t_0/t_0 \) where \( t_0 \) is the residence time of an unretained compound that permeates the particles) and the selectivity (\( \alpha = k_2/k_1 \)). Equation 2.12 and Fig. 2.3 demonstrate that the most effective way to increase chromatographic resolution is through changing the selectivity and choosing the appropriate separation mode based on the physicochemical properties of the analytes. On the other hand, the least rewarding parameter is evidently the optimization of \( N \) (longer column, smaller \( d_p \)) as
resolution follows a square-root dependency. Nevertheless, it is important to state that since $R_s$ differs for each specific peak pair it cannot be used as a generic metric to describe the quality of a separation but only that of two adjacent peaks.

**Figure 2.3.** Influence of the retention factor ($k$), the selectivity factor ($\alpha$), and efficiency ($N$), on chromatographic resolution. The blue line represents varying the selectivity (plate number and retention factor kept constant), the orange line represents varying the retention factor (plate number and selectivity kept constant) and the black line the plate number (selectivity and retention factor kept constant). The selectivity is seen to be the most rewarding factor to increase resolution, followed by the retention factor, but a large change in efficiency is required to increase the resolution. Adapted from [19].

### 2.3.3. Peak capacity in isocratic and gradient mode

Another way to measure the generic efficiency of a separation is peak capacity ($n_c$) which was defined by Giddings as the number of peaks that can be separated with unit resolution within the retention window [11] and can be expressed mathematically as:

$$n_c = 1 + \int_{t_0}^{t_R} \frac{1}{4\sigma} dt$$

(2.16)

In isocratic conditions where the $N$ (theoretical plate number) is commonly used to measure efficiency, $n_c$ is formulated as follows:

$$n_c = 1 + \frac{\sqrt{N}}{4R_s} \ln \left( \frac{1+k_\omega}{1+k_\alpha} \right)$$

(2.17)

where $k_\alpha$ and $k_\omega$ are the retention factors of the first and last eluting peak respectively [26]. On the other hand, $n_c$ is the most used metric for chromatographers when gradient elution is applied because in this case $N$ is difficult to be determined via the temporal band variance ($\sigma_t$) as the retention factor at the moment of elution ($k_e$) is always smaller than the observed $k$ and hence cannot be directly measured [27]. It can be calculated using the following equation:

$$n_c = 1 + \frac{t_G}{W_b}$$

(2.18)

where $W_b$ is the peak width measured at the baseline and $t_G$ is the gradient time, in which case the value is calculated for the whole elution span. Nevertheless, in many cases the elution
window is not covered entirely by peaks and thus the peak capacity value is overestimated and hence incorrect. When this is the case, the sample peak capacity must be used and the $t_G$ should be replaced by the difference in elution time between the most retained and the least retained analyte.

### 2.4. Unidimensional chromatography

#### 2.4.1. Optimization of unidimensional separations

The use of the aforementioned efficiency metrics permits the comparison of columns or methods, as well as the evaluation of parameters like the column length, particle size, temperature or the applied backpressure that can influence a separation. The goal is to find an optimal combination that would increase the efficiency while keeping the analysis time as low as possible, a condition that represents the classic trade-off that every chromatographer has to face. A few of the pioneers of the field have proposed the use of plots for the visualization and comparison of the performance limits starting with Giddings in 1965 who did that for gas chromatography (GC) and HPLC in terms of plate number $(N)$ and column dead time $(t_0)$ which of course reflects the total analysis time [28]. Many years later, in 1997, this concept was revisited and refined by Poppe who devised a plate time $(t_0/N)$ versus plate number $(N)$ plot, in order to gain an enhanced view of the $C$-term dominated region [28]. In the mid-2000s, Desmet et al. developed an approach called the kinetic performance limit (KPL) method, based on extrapolation, for the visualization of the kinetic limits and direct comparison of different columns or methods [29]. The construction of a kinetic plots starts from recording the same data needed for a Van Deemter plot, namely the efficiency in terms of $H$ (or $N$) for a range of linear velocities $(u_0)$ at a fixed column length. Following, the free-length kinetic plot can be constructed as an extrapolation of these values at the maximum backpressure ($\Delta P_{\text{max}}$) by using the following equation in the case of isocratic elution [29]:

\[
N_{KPL} = \frac{\Delta P_{\text{max}}}{\eta} \left( \frac{K_{v,0}}{u_0 \eta} \right)^{\text{exp}}
\]

\[
t_{0,KPL} = \frac{\Delta P_{\text{max}}}{\eta} \left( \frac{K_{v,0}}{u_0^2} \right)^{\text{exp}}
\]

where $K_{v,0}$ is the column permeability and $\eta$ the viscosity of the mobile phase. The index $\text{exp}$ stands for experimentally obtained values. The resulting plot presented in logarithmic axes (Fig 2.4) consists of a series of points that represent an extrapolation at the maximum available system back pressure of each experimental $t_0$, $N$ value obtained on a fixed column length, while
keeping \( u_0 \) constant. Considering the fixed \( u_0 \) value, the column length is the only available parameter that can be changed in order to reach the maximum back pressure hence the points on the KPL curve also correspond to the maximum achievable column length for each flow rate (or \( u_0 \)).

![Figure 2.4](image)

**Figure 2.4.** Illustration of kinetic plot curves at different column lengths leading to the kinetic performance limit curve (grey line) by extrapolation of the data on a single column length. The full black line represents experimental data measured on a 15cm column and the dotted black lines represent hypothetical data at different column lengths. The KPL line passes thought the maximum available pressure point and hence efficiency for all the curves. Taken from [30].

In a similar way, when gradient elution is considered, KPL can be constructed this time using peak capacity as a performance measure, instead of the plate number, for the reasons explained in “Section 2.3.3”. According to Broeckhoven et al. for the KPL calculations to be valid under gradient elution conditions, some assumptions must be made, namely that the \( \eta, H \) and effective retention factor (\( k \)) should be independent of the column length \( L \) [27]. For this to hold true, the necessary and sufficient condition is that the analytes are experiencing the same “relative mobile phase history” which in practice means that the gradient steepness \( \beta \cdot t_0 \) should be kept the same regardless of the \( L \) or the applied flow rate, where \( \beta \) is expressed as the change in organic modifier over the gradient time (\( \Delta c / t_G \)) [27]. In the case of gradient chromatography, the extrapolation of the KPL data from the experimental ones can be performed in a different manner using the so-called elongation factor \( \lambda \), that is the ratio between the maximum available system pressure and the experimentally measured column pressure at each different applied flow rate, via the following equations [27]:

\[
\lambda = \frac{\Delta P_{\text{max}}}{\Delta P_{\text{exp}}} \tag{2.21}
\]

\[
n_{c,KPL} = 1 + \sqrt{\lambda} \cdot (n_{c,\text{exp}} - 1) \tag{2.22}
\]

\[
t_{R,KPL} = \lambda \cdot t_{R,\text{exp}} \tag{2.23}
\]
At this point it is important to state that the method using the elongation factor $\lambda$ to extrapolate the KPL values is also valid for the calculations in the case of isocratic separations. This can be readily proven by inserting Eq. 2.1 in Eqs. 2.16 and 2.17, where after a few simplifications the ratio $\Delta P_{\text{max}}/\Delta P$ appears again.

### 2.5. Multi-dimensional separations

In the quest of analytical scientists to tackle the issue of sample complexity, present in various fields but in a greater extent in biological sciences, it has been seen that the use of unidimensional chromatography cannot provide the needed resolving power. As calculated by Giddings and Davis using the following equations

\[
p = m \cdot e^{-m/n_c}
\]

\[
s = m \cdot e^{-2m/n_c}
\]

in the case of a sample with $m$ randomly spaced components, a chromatogram can contain a maximum number of peaks ($p$) equal to 37% of the calculated peak capacity and to make this condition worse only 18% of the value will represent singlet peaks ($s$) [31]. Despite the advances in LC with the use of improved particle technology (sub-2 μm, core-shell technology) and state-of-the-art pump instrumentation (1500 bar commercial systems) the gain has been more significant in terms of reduced analysis time rather than increased separation power [32]. Even when mass-spectrometric (MS) detection is used, the issue of sample complexity persists as the coelution of analytes can hinder the ionization efficiency and the wide concentration ranges impede the accurate deconvolution of the peaks. Additionally, when using MS, the chromatographer should take also into account a more limited range of potential buffer systems that provide the needed volatility to yield an efficient sample ionization. To reach the higher peak capacities needed, multi-dimensional chromatography (MD-LC) approaches were introduced making use of a series of independent separation mechanisms/techniques for the analysis of such complex samples. The origins of this approach can be traced back to thin layer chromatography (TLC) where a variation of it was conceived by Haugaard and Kroner in 1948 who applied voltages along the edges of the plate that are in parallel with the 1D-TLC separation which was instead performed in the middle of the plate, in order to make use of the whole plane of the TLC plate [33]. In a work by Kirchner et al., published in 1951 partition chromatography, without the application of voltages, was used in both dimensions for the separation of terpenes.
First, the sample was spotted on the edge of the plate which was placed in the chamber containing the mobile phase and the development was carried out, with the front displacing the analytes until it reached the end of the plate. After the first dimension ($^1$D) development the plate was dried, rotated $90^\circ$ and inserted in the chamber containing a different mobile phase to exploit different retention characteristics in the second dimension ($^2$D). More than twenty years later, in 1975, O’Farrell presented what is still the most impactful type of planar multi-dimensional separation when he introduced 2D gel electrophoresis where proteins are separated first by isoelectric focusing in the $^1$D and then by gel electrophoresis based on their size in the $^2$D [35].

Nevertheless, technological advances in the field of analytical instrumentation probed researchers to move their focus towards column formats and as a consequence, multi-dimensional separations followed the same trend. Erni and Frei in 1978 and a few years later Bushey and Jorgenson paved the way for automated column MD-LC with their comprehensive coupling of columns for gel permeation chromatography and RPLC for the former and RPLC with CZE in the latter case [36,37]. In 1995 Moore and Jorgenson described the first three-dimensional separation of peptides combining SEC/RP/CZE, further proving the advantages of increasing the dimensionality in separations but also discussing drawbacks like the rather large dilution factors and increased analysis times [38]. In column based MD separations, the set-up consists of a pump for every dimension (or high voltage power supply in case of electro-driven techniques), an injector, $i$-1 interfaces (where $i$ is the number of dimensions) consisting usually of an 8 or 10-port switching valve equipped with two loops that are used alternately to store the collected fractions from the outlet of the $^1$D and re-inject them in the $^2$D column, and finally a detector (Fig. 2.5).

![Figure 2.5](image_url)  
**Figure 2.5.** Flow paths for the two positions of an 8-port valve used in a conventional 2D-LC setup.
The sample is injected into the 1D column and the effluent containing the analyzed fractions is stored at the interface and transferred to the 2D to be subjected to the additional separation. Based on how this procedure is done, we can distinguish different types and implementations of MD separations.

2.5.1. Types of multi-dimensional separations

2.5.1.1. Comprehensive multi-dimensional separations

In comprehensive MD separations (for liquid chromatography denoted as LC×LC) the goal is to separate the whole range of analytes in the mixture (e.g., metabolomics or proteomics studies [39,40]), hence the entire effluent of 1D must be collected in fractions and injected into the 2D (Fig. 2.6). This collection of fractions when not performed properly, can reduce the obtained efficiency of the prior dimension and consequently of the overall separation as a result of the remixing process of partially separated fractions during the collection. To avoid or limit as much as possible this issue, the chromatographer is required to find an optimum period of fraction collection (see “Section 2.5.3”).

![Figure 2.6. Conceptual representation of a comprehensive 2D-LC separation. Adapted from [41].](image)

2.5.1.2. Heart-cutting multi-dimensional separations

When the goal is the analysis of a number of targeted analytes (e.g., target-analytes with very similar retention characteristics, only part of the sample is of interest) then a few peaks or fractions of a specific peak are collected at the outlet of one dimension and injected
in the following, and hence we can talk about heart-cutting separations, see Fig. 2.7 (denoted as LC-LC) [40].

![Figure 2.7. Conceptual representation of a heart-cutting 2D-LC separation. Adapted from [41].](image)

### 2.5.2. Implementation of multi-dimensional separations

#### 2.5.2.1. Online multi-dimensional separations

In online MD separations the columns of the different dimensions are operating simultaneously and the effluent from one dimension is injected in the following after it is collected via the loops mounted on the switching valve (see Fig 2.5). This means that online 2D platforms should ideally be implemented with a 1D that consists of a narrow-/micro-bore column aiming at a slow separation with maximum resolution for the given application, followed by a short and possibly wider-bore 2D column allowing for faster separations as the analysis time in this dimension is constraint to be equal to the sampling time [42,43]. The total analysis time in online MD separations is calculated to be equal to the time employed in the first dimension. Intuitively, one can understand that, in online 2D separations, in order to have a sufficiently high sampling frequency of the 1D effluent, the second dimension must be performed in short analysis times, a condition that significantly lowers the resolving power of each 2D separation [41]. Nevertheless, this type of multi-dimensional separations yields the fastest results and can be fully automated, being hence a very compelling option for routine analysis of complex samples.

#### 2.5.2.2. Offline multi-dimensional separations

The exact opposite approach, defined by the term offline (LC/x/LC), is when the dimensions of the MD separation are decoupled and the effluent fractions of one dimension are
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collected and stored before they are subjected to the following analysis. This naturally comes with the advantage of being able to optimize each dimension separately without having the sampling time constraint and also the possibility to resuspend the collected fractions in a mobile phase that matches the one intended to be used in the following separation [42]. Both conditions are very important and can surely increase the overall efficiency but come with great expense in the overall analysis time, which in this case can be calculated as the sum of the time employed to analyze each collected fraction in the last dimension. Moreover, the storage of samples in offline methods can sometimes lead to loss of trace species and/or decomposition of analytes. Overall, offline methods are more laborious than online ones and yield generally lower resolving power per time unit [41].

2.5.3. Efficiency of multi-dimensional separations

In multi-dimensional separations involving the combination of two or more retention mechanisms the most appealing characteristic is the association of each additional dimension with the “product rule”. This is a concept proposed by Giddings which states that the peak capacity of a MD-LC system is equal to the product of the individual peak capacities achieved in each dimension (2...i) as visualized graphically in Fig 2.8

\[ n_{c,i} = n_{c,1} \times n_{c,2} \times \ldots \times n_{c,i} \]  

(2.26)

provided that “in each step the displacements depend on different factors” and that “two components that are substantially separated in any single step, they remain separated until the completion of the separation step” [44].

**Figure 2.8.** Schematic representation of the product rule for peak capacity calculation in 2D separations. Each box represents a unit of peak capacity. Taken from [41].
The above two statements mark two crucial factors that can influence the efficiency in a multi-dimensional system. The first is the adequacy of the combination of separation mechanisms in the consecutive dimensions. When considering the number of different separations mechanisms and modes (presented in “Sections 2.1” and “2.2”) that can be implemented, one can readily understand that there can be multiple combinations, but not all of them are suitable for a MD platform [45]. Their degree of independency which has been described by the term orthogonality together with their mobile phase compatibility has been the focus of several published studies. Gilar et al. introduced a geometrical approach that uses the degree of surface coverage with peaks as a descriptor of orthogonality, after plotting the normalized retention data in a 2D separation space and assigning a peak to every data point [46]. Stoll worked on a variation of the Gilar approach where he divided the separation space in a grid based on the relative peak capacity of each dimension [47], drawing also a rectilinear outline of the covered surface in a similar manner with Davis’ minimum alpha hull idea [48]. As no combination of mechanisms in 2D-LC is likely to be considered absolutely orthogonal, these approaches offer the means to quantify the usage of the 2D separation space with the so-called surface coverage ($f_{coverage}$). Based on that, one could correct the theoretical peak capacity of a multi-dimensional system whenever the system deviates from orthogonality and hence from the product rule. The second very important statement has to do with the remixing problem caused by suboptimal sampling frequencies. Murphy et al. were the first ones to study this bottleneck in a more fundamental manner by modelling a gaussian peak of 8σ width as a histogram and found that three to four samples should be ideally taken to minimize remixing effects [49]. Davis together with the group of Carr introduced a quantitative undersampling factor <$\beta$> that can be used with the product rule to correct the total peak capacity of a MD system. This factor is a function of the average peak width obtained in the preceding separation ($1\sigma$) and the sampling time ($t_s$) as described by the following equation [50]:

$$<\beta> = \sqrt{1 + 0.21 \left(\frac{t_s}{1\sigma}\right)^2} \quad (2.27)$$

When using the above equation and also incorporating the correction factor $f_{coverage}$ accounting for the orthogonality of the chosen separation mechanisms in each dimension, the corrected total peak capacity of a two-dimensional separation platform ($n_{c,2D}$) can be calculated as [45]:

$$n_{c,2D} = \frac{n_{c1}^2 n_{c2} f_{coverage}}{<\beta>} \quad (2.28)$$

The effective peak capacity for each individual dimension ($n_{c1}$, $n_{c2}$), used in Eq. 2.28, can be calculated via the equations presented in “Section 2.3.3”. 
2.5.4. Pareto optimization approach

Optimization of MD platforms seeks the balance between resolving power and analysis time by choosing the most suitable system and method parameters. When considering the coupled characteristics of the first and second dimension in online comprehensive 2D-LC, in combination with the numerous factors that affect the separation efficiency, one can understand how laborious the optimization procedure can be. To overcome this problem there have been developed predictive and optimization tools that let the analyst reach optimal conditions with a reduced expense in time and resources. The need though to optimize multiple parameters at the same time, lead the interest towards multi-objective optimization approaches and specifically Pareto optimality, a concept first used in economics. Pareto optimization is a chemometric approach able to find a single expression that correlates all the different experimental criteria (objectives) that need to be optimized. An experiment is Pareto optimal when there is no other experiment which has better results on one objective without having worse result(s) on other(s). The set of all Pareto optimal solutions is called Pareto front (Fig. 2.9). This concept can be extended to more than two objectives but difficulties in the graphical interpretation of the outcome, make it less attractive.

![Graphical representation of Pareto optimality approach](image)

**Figure 2.9.** Graphical representation of Pareto optimality approach. The blue points represent 10 experiments yielding results as function of two objectives. When the optimization goal is to maximize objectives 1 and minimize objective 2, then the Pareto optimal allocations are the experiments number 2, 4, 6, 9, and 10 which dominate upon the other ones on one objective without deteriorating the other. The orange line shows the Pareto front that passes through all the optimal points.

With more than two objectives to be optimized simultaneously, it becomes less probable that one experiment will clearly dominate over the other for all the objectives and this makes the interpretation of the result more challenging [51]. Vivo-Truyols *et al.* first applied a Pareto
based approach for the optimization of two-dimensional liquid chromatographic separations having as objectives the maximization of peak capacity while reducing analysis time. The method, that can account for losses in peak capacity due to undersampling and high injection volumes, yielded optimal values on parameters such as particle size, column diameter and modulation time. Regarding the last one, it was found that the optimal number of cuts per peak was between 2 and 3 [52]. A few years later Pirok et al., based on the same approach, developed a program for the interpretive optimization of two-dimensional resolution. Starting from a linear retention model for each dimension, based on only two LC×LC experiments, the program predicts the chromatograms (based on a Van Deemter model) and optimizes various objectives such as the resolution, the analysis time and the orthogonality of an IEX × ion pairing RP separation [53].

2.6. References


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CHAPTER 3

DEVELOPMENT OF A GENERIC ULTRA-HIGH-PRESSURE GRADIENT LIQUID-CHROMATOGRAPHY METHODOLOGY FOR THE ANALYSIS OF ANTIBIOTICS IN FOOD PRODUCTS

Chapter based on the article:

Development of a generic ultra-high-pressure gradient liquid-chromatography method development protocol: The analysis of residual multi-class antibiotics in food products as a case study

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3.1. Introduction

Dairy and meat products from cows are consumed by the majority of the global population and require a supply in excess of 700 million tons yearly to maintain this demand [1]. A significant threat to milk production in dairy cows is colonization of the milk ducts by *Streptococcus aureus* bacteria (mastitis) which represents a significant economic hazard ($2 billion/year) [2,3]. Consequently, a variety of antibiotics are frequently used to prevent the spreading of infection in dairy farms [4]. In particular, the beta-lactam class as well as tetracyclines, fluoroquinolones, sulfonamides and aminoglycosides constitute a complex mixture containing a broad range of analytes that could potentially be present in food products [5]. While the economic benefit of the use of antibiotics is significant, they can have deleterious effects on human health and therefore represent an enormous risk [6]. In particular, the risk of antibiotic resistance in bacteria constitutes the main threat and it has been observed recently that antibiotic resistance genes can be found in the excrement of cattle treated with antibiotics [7]. As such, analytical methods which can quantify the maximum safe levels of these compounds are required to ensure the safety of the various food products [8]. Liquid chromatography (LC) hyphenated to mass spectrometry (MS) has emerged as the method of choice for the characterization and quantification of antibiotics in food products [9].

The introduction of commercially-available ultra-high-pressure (UHP-) LC instrumentation with dedicated column technology has significantly spurred the potential to increase throughput and/or improve the separation efficiency. Gradient UHPLC has the ability to separate analytes, such as antibiotics, that are characterized by a broad hydrophobicity range within a reasonable analysis time for an analytical quality control laboratory (∼1 h), while improving the separation of structurally related antibiotic classes. Moreover, the application of a solvent gradient induces peak compression as the peak front elutes slower than the peak tail, leading to a decrease in peak width and hence improved detectability. To fully utilize the resolving power provided by ultra-high pressure gradient LC, kinetic plots can be used to evaluate the quality of the separation and determine which combination of parameters (operating pressure, particle diameter, column length, and gradient steepness) results to the highest resolving power within the shortest analysis time, when operating at the limit of the instrumental operating pressure (kinetic limit).

In this communication, we report on gradient method development targeting the establishment of high-resolution ultra-high-pressure (1500 bar) 1D-RPLC gradient separations...
of multiclass antibiotics using columns packed with 1.5 µm core-shell particles. First, the effects of flow rate and gradient duration on resulting sample peak capacity and gradient occupancy (ω, defined as the ratio of the retention window and the gradient duration, i.e., \( \omega = \frac{t_{R,\text{last}} - t_0}{t_G} \), where \( t_{R,\text{last}} \) is the retention time of the most retained compound in the sample) were assessed. Following, we extrapolate the sample peak capacities to the kinetic performance limit of 1500 bar, allowing prediction of the optimal combination of column length and gradient steepness to achieve certain peak-capacity thresholds within the shortest possible analysis time. Finally, the simulated gradient performance was compared to experimentally derived peak-capacity values obtained in gradient LC mode utilizing serially coupled columns.

### 3.2. Materials and Methods

#### 3.2.1. Chemicals and materials

Acetonitrile (ACN, HPLC supra-gradient grade) and formic acid (FA, purity 99%) were purchased from Biosolve (Bornem, Belgium). Ultrapure (18.2 MΩ-cm⁻¹) water was generated from a Milli-Q filtration system (Merck-Millipore, Molsheim, France). Uracil (≥99%) was purchased from Sigma-Aldrich (Bornem, Belgium). Multi-class antibiotics (Sigma-Aldrich) were spiked in milk at a concentration of 0.05 mg·mL⁻¹. Sample 1 contains: marbofloxacin, ciprofloxacin, enrofloxacin, danofloxacin, difloxacin, sarafloxacin, oxolinic acid, flumequine, cinoxacin, dapsone, enoxacin, nalidixic acid, norfloxacin, desacytylecephaprin, amoxicillin, ampicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicillin G, penicillin V, cefalexin, cefalonium, cefapirin, cefazolin, cefoperazone, cefquinome, ceftiofur, desfuroyl ceftiofur cysteine disulfide, erythromycin, spiramycin, tilmicosin, tylosin, lincomycin, pirlimycin, 4-epi-chlortetracyclin, 4-epi-oxytetracyclin, 4-epi-tetracyclin, chlortetracycline, oxytetracycline, tetracycline, sulfachloropyridazine, sulfagloxine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfathiazole, sulfathiazole, sulfamethoxypyridazine, sulfapyridine, sulfadinoxaline, sulfathiazole, trimethoprim, chloramphenicol, thiamphenicol, doxycycline, gamithromycin and florfenicol. Sample 2 contains only: marbofloxacin, ciprofloxacin, enrofloxacin, danofloxacin, difloxacin, sarafloxacin, oxolinic acid, flumequine, cinoxacin, dapsone, enoxacin, nalidixic acid, norfloxacin and desacytylecephaprin.
3.2.2. Sample preparation

Milk samples were prepared as follows: 4 mL of milk was spiked with the antibiotics at a final concentration of 0.05 mg/mL by adding 200 µL of a 1 mg/mL stock solution of the antibiotics, according to the sample components list provided in previous section. After mixing, 6 mL of acetonitrile was added, and the samples were vortexed for 10 minutes. The mixture was centrifuged at 6800 g for 10 minutes and transferred to a graduated tube, where the extract was evaporated at 40 °C under nitrogen, until 4 mL remained. This extract was diluted 1:2 in aqueous mobile phase (0.1% formic acid) and incubated at 4°C for 10 minutes to precipitate the proteins, before being filtered through a 0.2 µm filter (Merck-Millipore, Molsheim, France).

3.2.3. UHPLC instrumentation and experimental conditions

Chromatography experiments were performed on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germering, Germany) consisting of a binary pump H, column compartment H, a split sampler HT, and a variable wavelength detector F equipped with a 45 nL flow cell. 75 µm i.d. nanoviper capillary tubing was used in the system. Injections were performed with a 0.5 µL injection volume, samples were kept at 4°C, the column compartment was maintained at 25°C in still-air mode and UV spectra were acquired at a wavelength of 245 nm at 50 Hz (0.1 s response time). Linear aqueous/acetonitrile gradients were applied (unless stated otherwise) with a fixed gradient span from 1 v% ACN to 72 v% ACN, containing 0.1% FA. Chromeleon version 7.2.8 was used for instrument control and data acquisition. 100 mm × 2.1 mm i.d. Accucore Vanquish C₁₈⁺ columns packed with 1.5 µm core-shell particles were purchased at Thermo Fisher Scientific (Vilnius, Lithuania).

3.3. Results and Discussion

3.3.1. Establishment of initial gradient conditions

When first developing a linear gradient separation, it is intuitively understood that a starting condition is required to optimize from. In this regard, Desmet and Blumberg [10] devised an equation to calculate the optimal mixing rate for linear solvent strength gradient separations, which we used to derive our starting condition:
\[ R_{\Phi, \text{def}} = \frac{\Delta c}{t_G} = \frac{0.5 \Phi_{\text{char}}}{t_0} \] (3.1)

where \( R_{\Phi, \text{def}} \) is the optimal default mixing rate for small molecules (MW < 1000 Da), \( \Phi_{\text{char}} \) is the characteristic strength constant equal to 1/S, with S being the slope of the logarithm of retention factor vs. the volumetric solvent composition calculated to be 6.8, \( \Delta c \) is the change in organic content of the gradient and \( t_G \) is the programmed gradient time. By rearranging Eq. 3.1 with respect to \( t_G/t_0 \) we can calculate that the optimal \( t_G/t_0 \) is 10 when operating at the isocratic optimal condition (0.4 mL/min). This corresponds to a \( t_0 \) time of 0.426 min and a \( t_G \) of 4 min for a 100 mm column packed with 1.5 \( \mu \)m core-shell C\textsubscript{18} particles, applying a gradient span (\( \Delta c \)) of 0.71. This value was determined from two initial scouting runs, starting with a \( \Delta c = 1 \) for the first one and adjusting the \( \Delta c \) based on the % of organic modifier at the moment of elution of the most retained analyte in the second run. Fig. 3.1 shows the corresponding separation of the complex antibiotics sample (sample 1) at these conditions using a previously optimized system configuration, with respect to extra-column dispersion [11]. The average peak width (W) measured at the tangential intercept of the baseline, corresponding to 13.4% of the peak height was determined to be 1.02 s (average RSD of 15.3%), yielding an overall sample peak capacity of 139. Peaks were first assessed for significant co-elution before being included in the calculation, with the averaging being performed on 2 peaks from the beginning, middle and end of the chromatogram to account for band broadening. Prior to performing further gradient runs, columns were tested in isocratic mode and only the ones yielding reduced plate heights below 2.5 were selected for this study.

![Figure 3.1](image-url)  
**Figure 3.1.** Separation of residual multi-class antibiotics (sample 1) performed on a 2.1 mm i.d. \( \times \) 100 mm column packed with 1.5 \( \mu \)m core-shell C\textsubscript{18} particles at a flow rate of 0.4 mL·min\(^{-1}\), applying a 6 min gradient (\( \Delta c = 0.71 \)). The column was maintained at 25 °C applying still-air column-oven configuration. UV spectra were acquired at a wavelength of 241 nm at 50 Hz (0.1 s response time).
3.3.2. Modelling the impact of isocratic efficiency and gradient volume on peak capacity generation

In a next step, a sample of reduced complexity (sample 2) was used to assess the peak capacity as a function of the flow rate, while systematically varying $t_G/t_0$ between 5 and 40 and applying a fixed $\Delta c$ (= 0.71). Fig. 3.2 shows the sample peak capacity when varying $t_G/t_0$ (Fig. 3.2A) and when varying $t_G$ (Fig. 3.2B), respectively. The sample peak capacity was calculated by averaging the peak widths of sample 2, taking only the peaks which did not suffer from excessive co-elution (at least 10 out of 14 peaks), while defining the separation space between the first and last peaks of the complex antibiotics (sample 1).

**Figure 3.2.** Effect of flow rate and gradient steepness in terms of $t_G/t_0$ in (A) and gradient duration ($t_G$) in (B) on resulting sample peak capacity. For (A) $t_G/t_0$ = 5 (black), 10 (orange), 20 (grey), 30 (blue), and 40 (yellow); for (B) $t_G$ = 2.5 min (diamonds), $t_G$ = 5 min (circles), $t_G$ = 10 min (triangles), $t_G$ = 20 min (squares). For both conditions $\Delta c$ was fixed at 0.71. The fittings were based on Eq. 3.2 applying $S = 6.8$, with $N = L/H$ and $A = 2 \, \mu m$, $B = 3.2 \, \mu m^2 \cdot ms^{-1}$, and $C = 0.7$ ms applying the Van Deemter equation.

Unless otherwise stated, we report sample peak capacity to prevent confounding results due to non-optimal gradient times. Averaging the peak widths across the gradient led to a standard deviation on peak capacity ranging between 7% and 23%. The trendlines displayed in Fig. 3.2
are based on Neue’s peak capacity ($n_c$) equation for small-molecule (MW < 1000 Da) analytes, defined as [12]:

$$n_c = 1 + \frac{\sqrt{N}}{4} \cdot \frac{1}{b+1} \ln \left( \frac{b+1}{b} e^{s \Delta c} - \frac{1}{b} \right)$$  \hspace{1cm} (3.2)

where $N$ is defined as the isocratic plate number, $S$ is the slope of the logarithm of retention factor vs. volumetric solvent composition and represents the sensitivity of an analyte to changes in organic composition, $\Delta c$ is the range of organic composition over the gradient, and gradient steepness $b$ is defined as:

$$b = S \cdot \Delta c \cdot \frac{t_a}{t_G}$$  \hspace{1cm} (3.3)

When fixing $t_G/t_0$, $S$, and $\Delta c$, changing the flow rate will directly correlate to the change in efficiency ($N$). $N$ represents the isocratic column performance defined by the classical A-, B-, and C-term contributions [13]. A, B, C values for columns yielding minimum plate heights around 2.2 $\mu$m were estimated to be 2 $\mu$m, 3.2 $\mu$m$^2$·ms$^{-1}$ and 0.7 ms, respectively. Fig. 3.2A shows that the flow rate yielding the maximum peak capacity lies between 0.2 and 0.4 mL/min in gradient mode, which concurs with the optimal van Deemter flow rate recorded in isocratic mode. Note, within the same trendline the gradient steepness does not change. Flow rates above the optimal led to a slight increase in C-term contribution, however, the magnitude is rather small because of the minimal path length afforded by the core-shell particles. Additionally, increasing $t_G/t_0$ (5, 10, 20, 30, 40) led to an increase in peak capacity. Data modelling with Eq. 3.2 by applying an average $S$ value of 6.8 largely coincides with the experimental data points extracted from the gradient LC experiments. The $S$ value was determined according to Snyder [14] via two gradient scouting runs where $t_G$ is varied. Fig. 3.2B depicts the effect of flow rate on resulting peak capacity when fixing the gradient duration ($t_G = 2.5, 5, 10$, and 20 min, respectively). For short gradients the maximum peak capacity is achieved when operating at high flow rate yielding almost 1500 bar operating pressure. In this case, the column is operated slightly above the optimal Van Deemter flow rate, but the C-term contribution to band broadening is negligible and consequently the gradient-steepness contribution (and more specifically $t_G/t_0$) in Eq. 3.2 dominates the sample peak-capacity generation at these conditions. Subsequently, as gradient steepness ($b$) is decreased ($t_G$ increased or $t_0$ minimized) the peak capacity is improved. A maximum peak capacity of approximately 210 is obtained operating with a 20 min gradient (within the range of experimental conditions applied). To reach the highest peak capacity values the column needs to be operated at slightly lower flow rates (close
to \( u_{opt} \)) corresponding to the Van Deemter optimal plate height as isocratic efficiency dominates the outcome. A moderate increase in sample peak capacity can be anticipated when increasing \( t_G \), however such a gain is only obtained at the expense of a longer analysis time, hence a greatly reduced peak-production rate (peak capacity per time unit). For example, looking in Fig. 3.2.B, at the flow rate of 0.3 mL/min, the data point for \( t_G = 2.5 \) min shows a peak capacity of around 90 while the one for \( t_G = 5 \) min shows a value of approximately 130. This means that doubling the gradient time does not double the obtained peak capacity hence we have as mentioned decreased peak productions rates. The same pattern can be observed for the rest of the points in the graph along the same flow rate. Again, good correlation (< 5% error) was obtained between the experimental data and the predictions based on Eq. 3.2. Note, the gradient occupation decreases with shallower gradients, leading to a slightly reduced peak capacity at high \( t_G \) and high flow rate in comparison to Neue’s \( n_c \) estimation. For \( t_G = 20 \) min with flow rate 0.5 and 0.6 mL/min no data were acquired as these corresponds to excessively high \( t_G/t_0 \) values (>40).

### 3.3.3. Extrapolation of kinetic limit performance

To maximize the resolving power in gradient mode, while effectively utilizing the maximum pressure provided by the UHPLC instrument, the kinetic performance limits were established (see Fig. 3.3), using the approach described in [15]:

\[
n_{c,KPL} = 1 + \frac{P^{\text{max}}}{P^{\text{exp}}} \cdot (n_{c,exp} - 1) \tag{3.4}
\]

\[
t_{G,KPL} = \frac{P^{\text{max}}}{P^{\text{exp}}} \cdot t_{G,exp} \tag{3.5}
\]

The subscripts “\( exp \)” and “\( KPL \)” refer to the experimental data points collected on a 100 mm long column applying different flow rates (fixing \( t_G/t_0 \)) and the corresponding data points on the KPL-curve, respectively. \( P^{\text{exp}} \) is the maximal pressure reached during the gradient run and \( P^{\text{max}} \) is the reference pressure for which the KPL-curve is established, in this case 1500 bar. This approach considers the effects of \( t_G/t_0 \) and \( N \) on resulting peak capacity and also the effect of column permeability. Fig. 3.3A shows the effect of varying \( t_G/t_0 \) on the resulting gradient kinetic performance limits while operating at 1500 bar and Fig. 3.3B provides the corresponding column-length information. The trendlines are merely a guide for the eye.
Figure 3.3. Gradient kinetic-performance-limit curves constructed via extrapolation of the of the sample peak capacity recorded on a 100 mm long column operating at different flow rates using Eqs. 5 and 6 for $t_G/t_0 = 5$ (black), 10 (orange), 20 (grey), 30 (blue), and 40 (yellow). (A) shows the KPL considering the peak capacity and gradient duration, (B) shows the corresponding column length required. The maximum operating pressure is 1500 bar and $\Delta c$ was fixed at 0.71.

Different regions can be distinguished in the kinetic plot where different $t_G/t_0$ optimally leverage peak capacity and analysis time. Generally, fast gradient separation using 100 mm short columns operated at 1500 bar and should be operated at $t_G/t_0 = 20$, see Fig. 3.4A for the corresponding chromatogram. Nevertheless, with complex samples like the one evaluated in this study, the achieved peak capacity (188) for a column of 100 mm even under optimized conditions is not enough to yield a sufficient resolving power. Indicatively, by using the equation from the statistical overlap theory (SOT) of Davis and Giddings [16], we found that a $n_c$ of 150 would fully resolve (as singlets) only 44% of the total number of peaks in the sample and a peak capacity of 200 would increase the % of singlet peaks to 54. For even higher values of peak capacity equal to 300 and 400, 66% and 73% of the total compounds would be fully resolved respectively. For the chromatography user, Neue [12] proposed a “rule of thumb” for estimating the necessary peak capacity according to $n_c = n^{1.5}$ where $n$ is the number of components in the sample mixture. To achieve the higher peak-capacity values that are needed,
longer columns should be applied, and $t_G$ should be increased accordingly, to operate at the kinetic performance limits.

**Figure 3.4:** Separation of 61 residual multi-class antibiotics (sample 2) performed on one $2.1 \times 100$ mm (A), two coupled $2.1 \times 100$ mm (B) and four coupled $2.1 \times 100$ mm (C) column(s) with 1.5 $\mu$m core-shell particles, utilizing $t_G/t_0 = 20$, $t_G/t_0 = 20$, and $t_G/t_0 = 40$ respectively, operating at the kinetic limit (1500 bar) with flow rates of 0.5, 0.235, and 0.12 mL min$^{-1}$ respectively. The column was maintained at $25^\circ$C applying a still-air column-over configuration. UV spectra were acquired at a wavelength of 241 nm at 50 Hz (0.1 s response time).

This involves the use of coupled-column systems, *i.e.*, from 200 mm long columns applying $t_G/t_0 = 20$ (see Fig. 3.4B) up to 400 mm long columns applying $t_G/t_0 = 40$, (see Fig. 3.4C) using zero-dead volume connectors for column coupling. We can observe (Table 3.I) that the kinetic extrapolation utilizing a 200 mm coupled column operated at $t_G/t_0 = 20$ predicted a peak capacity of $\approx 300$, while experimentally 283 was determined. For a coupled column system of 400 mm long (4 coupled columns) with $t_G/t_0 = 40$, a peak capacity of $\approx 400$ was predicted using the KPL extrapolation while we obtain an experimental value of 379. When applying the gradient duration predicted in the gradient kinetic plots to maximize peak capacity, the $t_G$ is considerably longer than the retention time of the most retained compound (especially when utilizing the coupled-column system in combination with shallow gradients, high $t_G/t_0$) and this is due to an unfavorable gradient occupancy.
### Table 3.1. Comparison of predicted peak capacities for different coupled column lengths at the kinetic performance limit vs the experimental values.

<table>
<thead>
<tr>
<th>$L_{col}$ (mm)</th>
<th>$\Delta P_{exp}$ (bar)</th>
<th>$n_{exp}$</th>
<th>$n_{c,KPL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 100</td>
<td>1500</td>
<td>188</td>
<td>200</td>
</tr>
<tr>
<td>2 × 100</td>
<td>1472</td>
<td>283</td>
<td>300</td>
</tr>
<tr>
<td>4 × 100</td>
<td>1493</td>
<td>379</td>
<td>400</td>
</tr>
</tbody>
</table>

#### 3.3.4. Impact of gradient parameters on utilization of separation space

When increasing gradient time (working at fixed flow rate) and when increasing the flow rate (operating at fixed gradient $t_G$) we observed a decrease in gradient occupancy. Furthermore, as expected, a decrease in occupancy is obtained with increasing $\Delta c$. The effect of $\Delta c$ on gradient occupancy is straightforward, as the organic concentration at any time point will be higher with increasing $\Delta c$, thus the elution for any analyte will be expedited and the remainder of the gradient duration will be unused. The relationship between the flow rate (modulation of $t_0$) and the $\omega$ can be explained as follows; when decreasing $t_0$ (either by reducing the column length or increasing the flow rate), more volume of mobile phase will pass through the column carrying the analyte band further along. The faster migration of the band results in earlier arrival at the column outlet, rendering a shortened retention window with respect to the gradient time. The dependency of $\omega$ on $t_G$ nevertheless is not straightforward. With increasing $t_G$ the retention window, calculated as the difference between the $t_0$ marker and the last eluting compound increases as shown by Eq. 3.5

$$\Delta t_R = t_R - t_0 = \frac{t_G}{S\Delta c} \ln \left( \frac{S\Delta c}{t_G} \cdot k_0 + 1 \right)$$  \hspace{1cm} (3.5)$$

where $k_0$ is the isocratic retention factor of the most retained compound when the gradient starts. However, as gradient time approaches infinity (closer to isocratic elution), the retention time of the last eluting compound begins to approximate the isocratic retention time.

$$\lim_{t_G \to \infty} \Delta t_r(t_G) = \lim_{t_G \to \infty} \left[ \frac{t_G}{S\Delta c} \ln \left( \frac{S\Delta c}{t_G} \cdot k_0 + 1 \right) \right] = k_0 \cdot t_0$$  \hspace{1cm} (3.6)$$

Therefore, while the retention window increases to an asymptote the $t_G$ increases linearly. Subsequently, the occupancy will decrease.
Theoretically, the gradient occupancy can be expressed as a function of both $t_0$, $t_G$, and $\Delta c$ based on Snyder and Neue’s gradient retention model [12,14].

$$\omega = \frac{1}{S\Delta c} \cdot \ln \left( \frac{S\Delta c t_0}{t_G} \cdot k_0 + 1 \right)$$  \hfill (3.6)

The partial derivatives of $\omega$, with respect to $t_0$, $t_G$, and $\Delta c$, help to understand the variation of $\omega$ as these operational parameters change. The partial derivative of $\omega$ against $t_0$ is expressed as:

$$\frac{\partial \omega}{\partial t_0} = \frac{k_0}{S\Delta c \cdot k_0 \cdot t_0 + 1} > 0$$  \hfill (3.7)

Since $S$, $\Delta c$, $k_0$, $t_0$ are all positive variables, $\omega$ decreases as $t_0$ decreases. The partial derivative of $\omega$ against $t_G$ is expressed as:

$$\frac{\partial \omega}{\partial t_G} = -\frac{t_0 \cdot k_0}{t_G \cdot (S\Delta c \cdot k_0 \cdot t_0 + t_G)} < 0$$  \hfill (3.8)

Since $S$, $\Delta c$, $k_0$, $t_0$ are all positive variables, $\omega$ decreases as $t_G$ increases. The partial derivative of $\omega$ against $\Delta c$ is expressed as:

$$\frac{\partial \omega}{\partial \Delta c} = \frac{t_0 \cdot k_0}{\Delta c \cdot (S\Delta c \cdot k_0 \cdot t_0 + t_G)} - \frac{\ln \left( \frac{S\Delta c t_0}{t_G} \cdot k_0 + 1 \right)}{S\Delta c^2}$$  \hfill (3.9)

Considering that $S$, $\Delta c$, $k_0$, $t_0$ are all positive variables, we set $\beta = \frac{S\Delta c t_0}{t_G} > 0$ and the Eq. 3.9 can be rewritten as:

$$\frac{\partial \omega}{\partial \Delta c} = \frac{1}{S\Delta c} \cdot \left( \frac{\beta}{\beta \cdot \Delta c + 1} - \frac{\ln (\beta \cdot \Delta c + 1)}{\Delta c} \right)$$  \hfill (3.10)

Let us consider function $f(t) = \ln (t + 1)$, given $\beta \cdot \Delta c > 0$. By the mean value theorem, there exists $0 < t < \beta \cdot \Delta c$ such that,

$$\frac{f(\beta \cdot \Delta c) - f(0)}{\beta \cdot \Delta c - 0} = f'(t) \Rightarrow \frac{\ln (\beta \cdot \Delta c + 1)}{\beta \cdot \Delta c} = \frac{1}{t + 1}$$  \hfill (3.11)

Assuming $t$ such that: $0 < t < \beta \cdot \Delta c$, we have $1 < t + 1 < \beta \cdot \Delta c + 1$ and $1 > \frac{1}{t + 1} > \frac{1}{\beta \cdot \Delta c + 1}$ so we have

$$\frac{1}{\beta \cdot \Delta c + 1} < \frac{\ln (\beta \cdot \Delta c + 1)}{\beta \cdot \Delta c} \Rightarrow \frac{1}{\beta \cdot \Delta c + 1} - \frac{\ln (\beta \cdot \Delta c + 1)}{\beta \cdot \Delta c} < 0$$ \hfill (3.12)

By multiplying Eq. 3.12 with $\frac{\beta}{S \Delta c}$ we have:

$$\frac{1}{S \Delta c} \cdot \left( \frac{\beta}{\beta \cdot \Delta c + 1} - \frac{\ln (\beta \cdot \Delta c + 1)}{\Delta c} \right) < 0$$ \hfill (3.13)
thus \( \frac{\partial \omega}{\partial \Delta c} < 0 \), which indicates that \( \omega \) decreases as \( \Delta c \) increases.

Furthermore, since the goal is to achieve a full utilization of the gradient window the left-hand side of Eq. 3.6 should be equal to 1, this expression can be used to determine the adjusted \( \Delta c \) value (\( \Delta c_{100\%} \)) allowing the gradient span to be fully utilized, reaching maximum gradient occupancy, for any given flow rate and gradient time (provided \( S \) and \( k_0 \) are known [14]). Conversely, the practicing chromatographer may also set the retention time of the last eluting analyte to be \( t_G \) and adjust the programmed range of organic composition to the \( \%B \) at the time of the elution of \( t_{R\text{last}} \) to maintain gradient steepness and reduce cycle time between samples. To maximize thus the throughput, Eq. 3.3 hence needs to be considered. Consequently, for a four coupled column system with \( t_0 = 5.46 \) min, \( t_G \) should be fixed at 134 min, while applying \( \Delta c = 0.49 \) as can be seen in Fig. 3.4 C.

### 3.4. Concluding remarks

Herein, we report the use of kinetic-plot modelling to optimize the separation of complex antibiotics samples through the use of state-of-the-art fused core-shell particle columns operated at the kinetic limit of 1500 bar. Scouting runs utilizing a 100 mm column allowed for the generation of free-length kinetic plots, which predicted the expected peak capacities as well as analysis times when elongating columns to reach the kinetic limit. Predictions concurred with experimental data. Using four 100 mm long coupled columns and applying the following conditions \( t_G/t_0 = 24.5 \), with a \( \Delta c = 0.49 \), and a flow rate of 0.12 mL/min, a maximum peak capacity of 379 could be generated, allowing to resolve 71\% of complex mixture containing 61 antibiotics. The current instrument set-up did not allow to significantly increase column length to further advance resolving power. Alternatively, when the resolving power provided by 1D-UHPLC is not sufficient (as in this case for the complex mixture of antibiotics), the use of two-dimensional liquid chromatography, utilizing orthogonal separation modes in 1st and the 2nd dimension is recommended. In the case of antibiotics which suffer from a large number of isobaric confirmatory MS fragment ions any gain in the peak capacity increases the likelihood of being able to discriminate between structurally related impurities and highlights the unique benefits of optimizing the gradient LC method.

Finally, we have confirmed the effect of modulating various gradient parameters on the gradient occupancy in 1D-UHPLC and subsequently provided a tool in which the optimal \( \Delta c \)
can be determined. As such, we provide below an optimal method to design and optimize
gradient separation conditions for coupled-column systems:

1) Based on the estimated complexity of the analytes (number of components \( N \) in
sample), use the Neue estimation approach (\( n_c = n^{1.5} \)) to determine the peak
capacity needed for unidimensional separation.

2) Based on two experimental scouting runs where only gradient time \( t_G \) is varied,
one can estimate the \( S \) and \( k_0 \) value by applying linear solvent-strength (LSS)
model proposed by Snyder [14].

3) Determine column void volume (\( V_0 \)) via injection of an unretained marker (e.g.,
thiourea at high ACN content) and correcting for the residence time in the
connection tubing.

4) Construct free-length gradient kinetic plot with a series of gradient steepnesses
\( (t_G/t_0: 5, 10, 20, 30, 40) \) with an arbitrary but reasonable \( \Delta c \) value (determined
from two initial scouting runs, starting with a \( \Delta c = 1 \) for the first one and
adjusting the \( \Delta c \) based on the % of organic modifier at the moment of elution of
the most retained analyte in the second run). Then from the peak capacity
determined in step 1, simultaneously extrapolate the optimum column length,
gradient time, and flow rate, to provide the required resolving power at the
shortest possible analysis time.

5) Solve Eq. 3.3 after setting \( t_G \) to be equal to the retention time of the last eluting
analyte in the optimized separation, \( t_0 \) as obtained from the above experiment
while maintaining the \( b \) constant. The optimized \( \Delta c \) is obtained to assure full
utilization of the gradient window.

3.5. References

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CHAPTER 4

TOWARDS SPATIAL COMPREHENSIVE THREE-DIMENSIONAL LIQUID CHROMATOGRAPHY

Chapter based on the article:
Towards spatial comprehensive three-dimensional liquid chromatography: a tutorial review

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4.1. Introduction

The analysis of very complex sample mixtures constitutes a major analytical challenge and generally requires a high-resolution chromatographic separation prior to the detection of individual sample constituents for identification and/or quantification purposes. An example of an application area where sample complexity may hamper the analysis is proteomics research, e.g., biomarker discovery. Current quantitative proteomics approaches rely on liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) and typically allow to routinely assess more than 2000 proteins [1] and in some cases even more than 10,000 proteins [2]. A major limitation on quantitation by MS/MS sequencing is the extensive peak overlap inducing ion suppression [3,4]. Even with the introduction of ultra-high-performance liquid chromatography and using longer (500 mm – 1 m) columns packed with sub-2-micron stationary-phase particles the resolving power remains insufficient to tackle contemporary life-sciences sample mixtures. Peak capacity ($n_c$) is the most commonly used term when it comes to measuring the resolving power of a separation technique and is defined as the number of peaks that can be separated (from adjacent peaks at a specific resolution) into a path length or space provided by the separation method [5]. Since chromatographic retention follows a Poisson distribution, the number of separated compounds is only a fraction of the calculated peak capacity, and generally a great excess in peak capacity is required to realize a baseline separation of all the constituents in the sample [6].

Two-dimensional liquid chromatography (2D-LC) allows to significantly increase the peak capacity [7], provided that orthogonal separation mechanisms are used [8], although not up to a level that is required in modern proteomics. Furthermore, in a conventional 2D-LC set-up, fractions are sampled during the first-dimension development and analyzed sequentially, see Fig. 4.1A for the concept, which compromises the overall analysis time and hence the ability of the technology for screening of large numbers of clinical samples. Following this approach, the Jorgenson group established a three-dimensional liquid chromatographic (3D-LC) workflow coupling a size-exclusion chromatographic column via a modulator to a reversed-phase (RP-)LC column, which in turn was hyphenated to capillary zone electrophoresis (CZE) via a gated-injection modulation interface [9]. The resulting chromatogram was a 3D representation of the data-set by stacking the slices of data from 2D RPLC / fast CZE analysis, and the intensity of peaks was shown in inverted gray scale. Although a maximum peak capacity of 2400 was established, the sequential analysis of fractions remains a bottleneck when high-throughput screening of a multitude of samples is required.
Figure 4.1. Schematic representation of the concepts of on-line 2D-LC, where fractions are analyzed sequentially (A), and spatial 2D-LC, where the second-dimension development is performed in parallel, significantly reducing the overall analysis time (B).

In spatial chromatography, components are separated in the spatial domain with each peak being characterized by X and Y coordinates in a plane in spatial 2D-LC or by X, Y, and Z coordinates in a three-dimensional separation body in spatial 3D-LC. Furthermore, all fractions are injected simultaneously from one dimension to the following, and their development occurs in parallel, see Fig. 4.1B for the concept explained for spatial 2D-LC, leading to a substantial gain in analysis time compared to conventional multi-dimensional LC. The first application of a spatial liquid chromatographic separation was based on thin layer chromatography (TLC). After spotting the sample mixture at one of the corners of a TLC plate, two developments were carried out successively, first on one side of the plate and then (after a drying step) at the side which is perpendicular to the first one. The total spot capacity of the system in this case is the product of the spot capacities of each single development, provided that orthogonal separation mechanisms are being used. Ideally, orthogonality can be achieved using two different solvent systems during the subsequent developments [10–12]. Also, the use of bilayer plates has been reported, that integrates a narrow strip of one type of sorbent (e.g., silica) for the first-dimension (1D) development which is coated side-by-side with a broader strip of another sorbent (e.g., octadecyl-modified silica) for the second-dimension (2D) development [11,12]. In 1979 Tyihak et al. introduced overpressured thin layer chromatography (OPTLC) with the use of a totally closed and pressurized ultramicro chamber that prevents evaporation of the mobile phase [13]. This approach yielded higher efficiencies in longer plates and offered an even better base for more efficient 2D-TLC separations. An excellent review on possibilities and limitations in TLC has been published by Poole [11].

The concept of spatial comprehensive three-dimensional (3D-)LC was introduced by Guiochon and Beaver 1983 [14]. Using a pressurized spatial 2D-LC device as starting point, it
was envisioned that after finalizing a spatial 2D-LC separation and drying the permeable TLC plate, a 3D body can be placed on top of the plate allowing to develop the third-dimension (3D) stage [15]. Fig. 4.2A shows a schematic illustration of the concept, where different colors indicate the three separation stages. Note that the 1D, 2D and 3D separations are still performed sequentially, but all 1D and 2D fractions are separated at the same time, in parallel. Detection was envisioned by tomographic means or by a multiple array planar detector. Extremely high separation power may be realized given that the maximum peak capacity is the product of the three individual peak capacities (calculated by Eq. 2.26) provided that orthogonal separation mechanisms are utilized [8,14].

In 2011 the Schoenmakers group demonstrated a prototype apparatus targeting spatial two-dimensional liquid-phase separations in a step towards spatial 3D-LC [16]. Almost thirty years after the concept of spatial 3D-LC was introduced, a first prototype microfluidic device for spatial 3D-LC was presented by the Eeltink group working together with Schoenmakers, see Fig. 4.2B [17]. This chip device was equipped with an interconnected microchannel structure composed of a 1D channel, 16 parallel 2D channels and 256 channels for the 3D stage, utilized physical barriers and flow distributors to confine and control the flow, and integrated polymer-monolithic stationary phases.

![Figure 4.2](image-url)  
**Figure 4.2.** Schematic view of a system for spatial comprehensive three-dimensional liquid chromatography, adapted from [15]. (A) and first prototype microfluidic chip for spatial 3D-LC integrating interconnected channel structures, flow distributors and physical barrier to control and confine the flow during subsequent separation stages (B), taken from [17].
4.2. Assessing the separation potential of spatial 3D-LC

In 1959 Giddings and Keller laid the foundations for the description of spot distribution and size in one-dimensional thin-layer chromatography (1D-TLC) [18]. In this paper, equations for the calculation of spot area and length were derived using an asymmetric diffusion model where the axial and radial diffusion rates are unequal due to the kinetics of partition. In the late 70s and early 80s Guiochon and coworkers published a series of papers on the performance of TLC, based on the metric of spot capacity [19–21]. The spot size and shape were defined in function of the initial sample spot size, development length, plate characteristics and by taking into consideration the mass transfer kinetics and diffusion processes that control the spot length and width. According to these studies 1D-TLC can generate theoretical values up to 25 and observed spot capacities in the range of 10-20 [10,21]. Based on the obtained results, the same group of researchers advanced their concept towards two-dimensional (2D)-TLC by introducing equations for the calculation of spot capacity [22]. An important consideration made, was that the peak capacity will be smaller than the product of the spot capacities of the first- and second-dimension developments given the fact that when the second development starts the spots have already a certain size, originating from the radial diffusion of the analytes during the first development. The results demonstrated that with 2D-TLC it is very easy to obtain spot capacities in the range of 100-250 but very difficult (and only under optimal conditions) to reach 400 [11,22].

In 1983, Guiochon et al. derived equations to describe the separation potential of spatial 2D and 3D-LC [14]. A distinction was made between separation systems that were operated either in the space-based domain (\(x_{\text{LC}} \times x_{\text{LC}} \times x_{\text{LC}}\) for spatial 3D-LC) or where the last dimension was operated in a time domain (\(x_{\text{LC}} \times x_{\text{LC}} \times t_{\text{LC}}\)). Fig. 4.3 shows the predicted peak capacity that can be generated in spatial \(x_{\text{LC}} \times x_{\text{LC}} \times t_{\text{LC}}\) as function of retention factor. The calculations were made considering the use of 10 µm, 7 µm, 5 µm, and 3 µm particles in a 100 × 100 × 100 mm\(^3\) separation space, using Eq. 4.4 from Table 4.I. Peak capacity increases quickly at the beginning with increasing retention factor \(k\) in all cases but even more rapidly with increasing the ratio of column length to particle size. The steepness of the curves starts decreasing for values higher than 10 implying that there is a limit at large \(k\). In fact, according to Guiochon et al. at very large \(k\) it is almost not feasible to achieve higher efficiency in isocratic \(x_{\text{LC}} \times x_{\text{LC}} \times t_{\text{LC}}\) than in \(x_{\text{LC}} \times x_{\text{LC}} \times x_{\text{LC}}\) for a device with the characteristics described above [14]. Theoretical predictions show that peak capacities of several tens of thousands and even up to 100,000 are considered possible in spatial 3D-LC [14]. In Table 4.I, a summary of the derived
Table 4.I. Simplified equations for multidimensional spatial chromatography with identical column characteristics in all dimensions (channel length ($L$), plate height ($H$), diffusion coefficient ($D$), and the mobile-phase velocity ($u$)) and assuming negligible size of the initial spot. In the reported equations $a = \frac{2\gamma}{hv}$, where $\gamma$ is the obstruction factor and $h$ and $v$ are the reduced plate height and velocity [14].

<table>
<thead>
<tr>
<th>Development in all dimensions</th>
<th>Spatial 2D-LC</th>
<th>Spatial 3D-LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n^{2D} = \frac{L}{4H} \left( \sqrt{1 + \alpha} - \sqrt{2\alpha} \right)^2$</td>
<td>(4.1)</td>
<td>$n^{3D} = \left( \frac{L}{H} \right)^{3/2} \frac{1}{8} \left( \sqrt{1 + 2\alpha} - \sqrt{2\alpha} \right)^3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Elution in the last dimension</th>
<th>Spatial 2D-LC</th>
<th>Spatial 3D-LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n^{2D} = \frac{L}{8H\sqrt{1 + \alpha}} \left( 2\sqrt{1 + \alpha(1 + k) - 2\sqrt{1 + \alpha} - 2\sqrt{\alpha(1 + k)} + \ln \frac{(\sqrt{1 + \alpha} + 1)[\sqrt{1 + \alpha(1 + k)} - 1]}{(\sqrt{1 + \alpha} - 1)[\sqrt{1 + \alpha(1 + k)} + 1]} \right)$</td>
<td>(4.3)</td>
<td>$n^{3D} = \frac{L^{3/2}}{16H^{3/2}\sqrt{1 + 2\alpha}} \left( 2ak' + \sqrt{8a(1 + 2a)} - \sqrt{4a[1 + a + (1 + 2a)(1 + k) + a(1 + k)^2] - (1 + 2a)} \cdot \ln \frac{2\sqrt{a[1 + a + (1 + 2a)(1 + k) + a(1 + k)^2] + 2a(1 + k) + 1 + 2a}}{(1 + k)\sqrt{2a(1 + 2a) + 1 + 4a}} \right) + \sqrt{4a(1 + a)} \cdot \ln \frac{2\sqrt{1 + a}[1 + a + (1 + 2a)(1 + k) + a(1 + k)^2 + 1 + 2a]}{(1 + k)\sqrt{2(1 + a) + 1 + 4a}}$</td>
</tr>
</tbody>
</table>
equations is presented for the simplified case where sample spot size ($\sigma_i$) is negligible and the plate characteristics (i.e., channel length, plate height, diffusion coefficient, and mobile-phase velocity) are the same in all dimensions. Nevertheless, this might not be the same as in the case of modern spatial 2D and 3D-LC systems where different selectivities as well as column lengths are intended to be used in the different developments.

![Figure 4.3](image.png)

**Figure 4.3.** Plot of the potential peak capacity in spatial 3D-LC ($x_{\text{LC}} \times x_{\text{LC}} \times t_{\text{LC}}$) as function of the retention factor at which the analysis is stopped. Calculations were made considering a pressure-driven development in isocratic elution mode with a three-dimensional separation space of $100 \times 100 \times 100$ mm$^3$ and the use of (1) particle size ($d_p$) = 10 µm, (2) $d_p$ = 7 µm, (3) $d_p$ = 5 µm, (4) $d_p$ = 3 µm particles with $A = 1$ and $C = 0.03$, and (5) $d_p$ = 5 µm with $A = 0.7$ and $C = 0.01$. Reduced velocity $\nu = 14$. Taken from [14].

The Schoenmakers group investigated the separation potential of (spatial) 2D- and 3D-LC using a Pareto optimality approach [23-25]. In these studies, maximizing peak capacity and minimizing analysis time were set as the objectives, while operating the device at high-pressure conditions (50 bar) [23,24]. Pareto fronts for spatial 2D-LC performed in the spatial domain ($x_{\text{LC}} \times x_{\text{LC}}$) were calculated based on the peak-capacity equation for isocratic separations derived by Guiochon [23]. This equation was also extended to incorporate a $^3$D development in gradient mode [24]. It was reported that Pareto fronts for $^3$LC × $^3$LC using a sorbent of TLC quality and operating a device at a maximum pressure of 50 bar yielded significantly better peak-production rates than a UHPLC × UHPLC system operating at 1000 bar in the time
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domain [23]. Davydova et al. reported that $^1\text{LC} \times ^1\text{LC} \times ^1\text{LC}$ performed best in terms of maximum achievable peak capacity in shorter analysis time but the main disadvantage of operating in the space-based domain is that analytes are retained in the separation body, which complicates detection [24]. In a follow-up study, Pareto optimal fronts were reported showing the total peak capacity as function of analysis time for the spatial 3D-LC device containing 16 $^2\text{D}$ channels and 256 $^3\text{D}$ channels depicted in Fig. 4.2B and a $^1\text{LC} \times ^1\text{LC} \times ^1\text{LC}$ separation was considered and compared to the case when the number of $^2\text{D}$ channels is doubled in combination with 1024 $^3\text{D}$ channels [17]. The latter chip design yielded almost a 3-fold increase in peak-production rate.

4.3. Chip design for spatial 3D-LC

4.3.1. General considerations

When effectively utilizing a three-dimensional separation body like the one depicted in Fig. 4.2A, the total peak capacity will be inherently higher than in the case where some of the available separation space is sacrificed by integrating microchannels. In the latter device however, flow control will be evidently easier to achieve, and the integrity of the subsequent separations will also be maintained once analytes are situated in the microchannel for the next development stage. Furthermore, in such a device with a discrete number of channels the contact points between the various substrate plates will be increased, yielding higher pressure resistance of the spatial chip. Moreover, it offers the possibility to incorporate electrical-driven separation modes which enhances not only its applicability but also eases the chip design with respect to flow confinement, see the discussion in “Section 4.3.2”. To achieve the highest possible peak capacity in spatial multi-dimensional LC it is essential to maximize the orthogonality during the different separation stages.

An important consideration in spatial LC is that the separations obtained during the $^1\text{D}$ and $^2\text{D}$ developments should be maintained inside the targeted space. As such, iso-electric focusing would be an obvious choice to be implemented as either $^1\text{D}$ or $^2\text{D}$ development in case of proteomics analysis. To make other separation modes applicable to spatial chromatography, one needs to ensure that the separation is terminated when the least retained analyte reaches the channel outlet. This may constitute a significant challenge, especially when taking into account that the most powerful modes for protein analysis are executed in gradient conditions, and that there is a steep dependency of the mobile-phase composition on the resulting retention factor.
For example, in gradient RPLC hydrophobic analytes will still be strongly retained while more hydrophilic analytes will already elute towards the column exit, making a spatial separation virtually impossible. However, to retain analytes at predetermined locations inside the separation channel it may be feasible to apply solvent modulation, ensuring that at the position where analytes need to be located, the solvent strength is (locally) adjusted. Alternatively, it may be possible to apply temperature or stationary-phase gradients creating a gradient in retention capacity along the channel length. Another possibility is to adapt the concept of field-flow fractionation to position peaks at specific locations in a microchannel or spatial device prior to sample transfer to the next development (see “Section 6.4.1” for a detailed discussion on the possibilities of different combinations of separation modes).

To analyze complex proteomics samples encountered in biomarker discovery studies it is important to scale the dimensions of the different development stages. The use of a large 1D i.d. channel format allows to maximize the mass loadability. During the 2D and 3D developments smaller channel dimensions are preferred to ensure flow rate compatibility when hyphenating the spatial device to mass-spectrometry detection. One approach envisioned for detection is to conduct the last development in time and deposit the resulting effluent on a membrane in regular time intervals, that can be subsequently analyzed via MS imaging technology, see the discussion in “Section 4.3.4”.

### 4.3.2. Flow control and confinement

Establishing homogeneous flows and confining the separation stages is critical to maximize the peak capacity, and to maintain the integrity (X, Y, Z coordinates) of the resulting separations. The requirements for chip design to deliver and confine flow during a specific development when utilizing pressure-driven flow are more stringent than in the case when electro-osmotic flow is utilized to propel the mobile phase. Independently of which mode is applied, the use of flow distributors can be considered to feed the mobile phase to the 2D and 3D separation stages.

The research group of Regnier was the first that demonstrated the concept of on-chip flow distributors and collectors to feed and collect the mobile phase from a wide-bore microfabricated chromatography column based on a collocated monolithic support structure (COMOSS), see Fig. 4.4A [30]. The channel network of the bifurcating flow distributor was designed such that the channel i.d. decreased after each level of bifurcation. Such distributor
was initially developed for electro-driven separations, but the same concept can be applied to distribute flow in pressure-driven mode. The designs of both bifurcating and radially-interconnected flow distributors were studied by the Desmet group using computational fluid dynamics (CFD) [31-34]. Vangeloooven et al. designed radially-interconnected flow distributors with diamond-shaped pillars having their longest dimension oriented perpendicular to the flow direction [31]. To achieve fast radial flow distribution over a short axial range, a zone of large-aspect ratio diamond shaped pillars was used, followed by a zone of small aspect-ratio diamond pillars to maximize the number of entrance points before reaching the separation stage, thus minimizing flow warping. These were validated by on-chip flow visualization in glass-etched chip. This design was successfully applied by Wouters et al. to develop the 2D stage using a microfluidic chip for spatial 2D-LC, see Fig. 4.4B [35]. CFD studies performed by Davydova et al. and Smits et al. yielded new insights in flow distributor design [33,36]. With respect to peak variance, the bifurcating flow distributors having a minimal volume gave the best dispersion characteristics. Recent on-chip flow-imaging experiments demonstrated that bifurcating distributors with an angle of 180° yield the most homogeneous flow profile (see Chapter 5 for the experimental results) [37]. When chip prototyping is the limiting factor and local imperfections (constrictions blocking a small segment of a microchannel) exist, a ‘hybrid’ flow distributor design can be utilized. This design is based on bifurcations but also integrates mixing zones after several levels of bifurcations (Fig. 4.4A and B) where different parallel zone flow paths come again in direct fluidic contact [34].

![Flow distributor designs](image)

**Figure 4.4.** Flow distributor designs that can be utilized either to feed the 2D stage (A and B) or the 3D stage (C and D) in a spatial 3D-LC separation. (A) depicts a bifurcating flow distributor reprinted with permission from [30]. (B) shows a radially-interconnected flow distributor with 2 zones for fast radial distribution of mobile phase and for homogeneous feeding of the 2D channels, reprinted with permission from [35]. (C) Fractal 3D flow distributor, reprinted with permission from [17]. (D) Design of a 3D flow distributor consisting of several bifurcating flow distributors connected with a main one at the top, taken from [39].

To feed the 3D separation stage composed of 256 3D microchannels in spatial 3D-LC chip, Wouters et al. integrated a fractal 3D flow distributor, see Fig. 4.4C [17]. This prototype
was derived from a design proposed by Tondeur et al. who described the theoretical concepts for a 3D flow distributor based on fractals [38]. The Schoenmakers group proposed a 3D flow distributor consisting of several bifurcating flow distributors connected with a main one at the top, see Fig. 4.4D for the channel layout [39]. CFD simulations showed that this design would prevent recirculation and mixing during the 2D development.

Various strategies can be pursued to confine the flow during the different separation stages. One can envision the development of a modular separation device where modules are fused together after completing the 1D and/or 2D separation stages. An example of a modular microfluidic device for 2D-gel electrophoresis was presented by Chen et al. [40]. This group developed a PDMS microfluidic system for a 1D development composed of 4 layers, shown in Fig. 4.5A. After completing the 1D development the top and bottom layers were removed (Fig. 4.5B), and two new layers were assembled allowing to execute the 2D development in parallel 2D channels, see Fig. 4.5C for the device layout.

Demianova et al. presented a modular device allowing to perform IEF followed by polyacrylamide slab gel electrophoresis (PAGE) [41]. An air slot was used to confine the flow during the 1D development. After completing the 1D run, the IEF and PAGE gel modules were pressed together, and the slot was passively filled with focusing gel. An advantage of performing macromolecule analysis is the slow diffusion rate, hence the integrity of the
separation after a development may only be compromised to a small extent while fusing modules together. Depending on material properties of the housing it may be also possible to cool the device after a development, further restricting the diffusion rate. Nevertheless, devices of such type might cause reproducibility issues due to manual errors.

In electro-driven spatial two-dimensional gel electrophoresis (2DGE) set-up, flow confinement can simply be realized by implementing multiple electrodes on chip and applying subsequent voltages for the different developments. Li et al. achieved separations with isoelectric focusing in a single channel during the 1D development and SDS gel electrophoresis in the 2D development using an array of 10 parallel microchannels [42]. Das et al. described flow confinement in 2DGE chip by local photo-polymerization of the gel for PAGE in the flow distributor and in the parallel 2D channels [43].

The use of physical barriers has also been pursued to confine the flow in pressure-driven spatial 2D- and 3D-LC. Wouters et al. constructed a microfluidic chip for spatial 2D-LC from different chip layers and compartmentalized the 1D separation by integrating the 1D channel in the top substrate while 2D flow distributor and channels were milled in the bottom substrate with the through holes connecting the 1D channel to the 2D channels having a cross-sectional area 10 times smaller than that of the 1D channel [35]. Flow confinement during the 1D development was demonstrated when applying external valves to block off the flow at the 2D inlet and outlet and in absence of stationary phases in the microchannels. Davydova et al. discussed size constrictions of 90% of the total area in the last row segments of a bifurcating flow distributor as means of creating preferential flow paths during a 1D development without recirculation of sample in the 2D flow distributor [44]. Restricting the cross-section areas further by integrating monolithic stationary phases locally in the flow distributors and 2D channels while maintaining an open 1D channel enhances flow confinement, as demonstrated by Liu et al. [45] and Themelis et al. [37]. From a CFD simulation, Adamopoulou et al. concluded that flow confinement in a pressure-driven spatial 3D-LC device may be achieved when using an open 1D channel and integrating high- and low-permeability stationary phases, with three orders of magnitude difference in permeability, in the 2D and 3D stages, respectively [39].

To confine flow during the 1D development, a concept based on ‘active valving’ was presented by the Schoenmakers group and by the Eeltink lab in 2018 [46,47]. In active valving, the flow during the 1D stage is confined within a cylindrical tube containing through-holes. These are either closed during the 1st development or opened after rotation of the tube when
aligned with the $^2$D flow distributor and the $^2$D channels, allowing for sample transfer and executing the subsequent $^2$D analysis. A chip prototype was developed using solvent-assisted drilling, in which a PEEK tube containing through-holes was drilled at low speeds into an existing small i.d. channel, present in a polymethylmethacrylate chip substrate while adding dichloromethane [37]. Adamopoulou et al. reported on a 3D printed device in which a dye was introduced in a transparent cylindrical channel and was directed towards the $^2$D channels after $90^\circ$ rotation [48]. A major challenge for such a device is to correctly align its components and at the same time create an interface tight enough to avoid leaks and be compatible with HPLC conditions. The through-holes present in the $^1$D tube must be properly aligned with the $^2$D flow distributor and $^2$D channels in order to allow the mobile phase to enter the $^1$D channel and sweep the analyzed fractions towards the second dimension. Themelis et al. reported on the development of a leak free microfluidic device for spatial 2D-LC, allowing to successfully confine the flow during the $^1$D stage via active-valving and establishing effective transfer of sample to the parallel $^2$D channels after rotation of the axis, by flow entering from the $^2$D flow distributor, feeding the parallel $^2$D channels (see Chapter 5) [37]. By using PEEK tube inserted in an elastic PFA sleeve and constructing a customized chip holder allowing to apply high vertical forces, no leak flow was observed when applying pressure as high as 50 bar. Furthermore, poly(methacrylate-co-ethylene dimethacrylate) monolithic support structures were synthesized in-situ in microchannels, allowing for stationary-phase focusing (when applying aqueous conditions) after sample transfer and a subsequent gradient reversed-phase separation, see also discussion in Chapter 5.

An alternative approach to confine flows in capillaries or microfluidic channels is by utilizing freeze-thaw valves [49]. This approach is generally limited to solvent systems containing high water content, as water expands during freezing. This concept was adopted by Nawada et al. who developed a titanium device containing a channel for the $^1$D stage, which was connected to parallel $^2$D channels oriented perpendicular to the $^1$D channel and portions of channels were enveloped by cooling and heating jackets [49]. The frozen plug was reported to withstand at least 200 bar of pressure. Switching times between a frozen state and a state with the section thawed for a single valve was found to be in the range of $1 – 2.5$ min using a passive switching approach, which could be reduced by a factor of 3 using an active switching (introducing hot liquid). A $58\%$ increase in switching time was observed when using a T-junction network of 33 of such valves instead of 1 [49]. In pursue of spatial 2D-LC separations, based on thin-layer chromatography, the Svec group used the dewetting properties of super
hydrophobic polymer-monolithic materials to confine an aqueous flow within a patterned hydrophilic channel functionalized with ion-exchange moieties during a 1D separation [50]. Using this approach, a mixture of peptides was separated based on ion-exchange chromatography while the aqueous mobile phase migrated only in the trench due to the large difference in surface tension at the interface of the hydrophilic channel and the superhydrophobic monolith. The unmodified hydrophobic layer was then used to develop the 2D stage based on RPLC interactions. The separation of peptides was monitored using desorption electrospray ionization coupled to MS detection.

Optimization of chip design is an important aspect to establish good sample transfer between the subsequent separation stages. Different studies have been conducted, to investigate sample transfer in a spatial 2D-LC configuration considering a parallel 2D channel structure and a flat bed, respectively [44,51,52]. The flat-bed approach may lead to better analyte recovery and a higher peak capacity [51]. When applying a parallel channel structure, the array of microchannels of bifurcating flow distributor can be aligned with the parallel 2D channels or shifted by half the distance such that the 2D channels are located between the exits of the flow distributor. Yang et al. conducted computational studies as well as on-chip flow visualization experiments to optimize sample transfer in microfluidic system a for spatial 2D-LC and concluded that the latter channel configuration is more efficient [52]. The use of an angled (zigzag) first-dimension channel has been shown to substantially improve sample transfer performance. Also, the number of out- and inlet points that are used to transfer the fractions, from one dimension to the following, is important. The smaller the distance between these points, the better will be the sampling. In an ideal situation, the distance would be infinitely small and the number of channels very high, reaching thus a quasi-flat-bed condition. If an analogy has to be made with the sampling rate in conventional column-based 2D-LC then that would dictate that a peak has to be sampled three to four times [53]. This value comes as a result of the compromise between good analyte recovery and acceptable analysis times in the 2D in conventional 2D-LC, where the limiting factor is the sampling rate which as becomes higher the 2D analysis should be faster a fact that usually leads to lower resolution. In spatial chromatography this limiting factor does not exist, as the different dimension separations are decoupled, and they are carried out sequentially which means that the sampling frequency does not affect the analysis time of the following dimension. Consequently, in such case the higher the sampling rate applied (higher number of channels in the following dimension), the better the analyte recovery is, up to a certain value where 100% recovery is reached, and no more
fractionation is necessary (see discussion in “Section 6.2.4” and “Section 6.4.3”). This means that in a dimension performing an efficient separation, where peak widths might be at the order of a few hundred micro-meters, the expected distance between two consecutive channels of the following dimension should be ideally at the order of tens of micro-meters. Such structural specifications are challenging, and special manufacturing conditions are required in order for them to be met. In case the spacing between the channels is not optimized, the transfer from one dimension to the following will lead in loss of resolution, band remixing and a loss in peak capacity, that could be partially compensated if a focusing effect at the head of the following dimension channels is introduced.

4.3.3. Utilization and incorporation of stationary phases

To establish spatial multidimensional LC separations, orthogonal separation modes need to be realized. This implies that most of the times stationary phases need to be integrated in the separation space/channels. A relatively straightforward approach to generate specific selectivities in-situ is to modify the composition of the mobile-phase during the different separation stages to exploit different properties of a mixed-mode stationary-phase surface. Conventional C18 modified silica stationary phases used in RPLC may display to some extent ion-exchange (IEX) behavior due to the presence of free residual silanol groups [54]. Mixed-mode materials are designed to enable dual and even ternary separation mechanisms based on the composition of the mobile phase and analyte properties, while maximizing mass loadability [55]. A wide variety of mixed-mode chemistry materials have been developed, and both particle-based and monolithic materials exist [56–60] An overview of the different chemistries and their applications have been summarized by Zhang et al. [55]. The most common chemistry combination is RPLC materials that also contain cation or anion exchange moieties. Also, mixed-mode materials have been developed allowing to establish HILIC selectivity, in combination with RP or IEX selectivity [59]. In 2011, Liu et al. introduced a trimodal stationary phase carrying RPLC, cation- and anion-exchange functionalities. The resulting selectivity is a complex interplay of physicochemical properties (e.g., van der Waals surface area, logP, and pKₐ characteristics), stationary-phase and analyte properties as function of the mobile-phase composition, i.e., the ionic strength, the pH, and/or by the type and content of organic solvent used [60]. With careful optimization of these parameters and by optimizing the pore size of the mixed-mode materials, also the combination of size-exclusion chromatography with (mixed-mode) interaction chromatography can be realized [61,62].
An alternative approach to locally generate different selectivities in a spatial separation device is by integrating responsive materials that can undergo reversible conformational changes after applying specific stimuli [63–65]. A well-known example of a thermo-responsive material frequently applied in separation science is poly(N-isopropylacrylamide) [66–68]. This material shows hydrophilic properties when operating below 32°C where polymer chains are expanded and above this temperature when chains are coiled, the polymer displays a hydrophobic character. This is an endothermic process and is driven by the gain in entropy which is associated with water molecules that lose orientation and dehydrate the polymer chain. Other stimuli-responsive materials include pH-responsive [69,70] and photo-responsive chromatography support structures [64], and also dual responsive materials, which retentive properties are affected by both temperature and pH yielding hydrophobic/hydrophilic, ion-exchange selectivity [71,72]. The configurations that may be of interest for spatial 3D-LC depend on how effectively stimuli can be applied. Thermo-responsive materials may be applicable in all three dimensions as solvents can be accurately controlled prior to chip entry, as column preheaters and post-column coolers are readily available. Ideally, the heat conductivity of the embodiment is preferably low to limit temperature gradients across the separation channel(s) and segments. To effectively utilize photo-responsive materials, transparent chip housing materials need to be employed and its use is likely to be restricted in the 1D or 2D separation stages, as the penetration depth of radiation may be limited.

Considering the difficulties encountered in conventional column manufacturing, incorporation of stationary phases in embodiments for spatial multi-dimensional chromatography is very challenging. Packing of a single separation body as depicted in the schematic in Fig. 4.2A with (mixed-mode) stationary-phase particles can be feasible, although channeling effects after closing off the device may be difficult to circumvent. Alternatively, synthesis of rigid monolithic stationary phases may be considered. Flat-bed polymer monolithic structures for spatial 2D-LC have first been developed by the Svec and Fréchet group as monolithic matrix for MALDI mass-spectrometry analysis [73,74]. Vanhoutte et al. described the development of a device from borofloat glass, which incorporated a flat polymer monolithic support structure for the 2D development [16]. After filling a trench with mixture of dyes, a separation was successfully carried out based on reversed-phase chromatography. Integrating a monolithic support structure in a 3D separation body spanning a larger cross section is challenging, as shrinkage of the support structure will occur during the polymerization reaction and once established, the high forces associated with swelling and shrinkage of polymer when applying a solvent gradient may still lead to monolith rupture. These problems can be overcome
by using a scaffold, albeit at the expense of available separation space, as used by Vonk et al. who developed a titanium-scaffolded polymer-monolithic structure compatible with UHPLC gradient operation in 4 mm i.d. column formats [75,76].

An alternative approach to a single 3D cube is to design a microfluidic chip for spatial 3D-LC characterized by a complex interconnected microchannel structure, as proposed by Wouters et al. [17]. Slurry packing of parallel channels with microparticulate materials may be feasible to some extent. For example, slurry packing of 12 parallel microchannels, relying on the keystone effect to retain particles in the microchannels, has been successfully demonstrated by Nagy and Gaspar [77]. Huft et al. established packing of 4 parallel 20 mm long microchannels [78]. Each separation channel was lined with 520 ‘by-pass channels’ equipped with Quake valves along the axial column direction, allowing the slurry solvent to flow laterally through the side (‘wall’) of the separation channel during the packing process. Following the packing, the by-pass channels were closed using the microvalves. The DeVoe group developed a microchip for IEF during the 1D development and transfer fractions to 5 parallel packed 2D channels for a following gradient reversed-phase LC separation [45]. Incorporating stationary-phase particles in a chip containing parallel 2D and a multitude of parallel 3D channels is inherently much more difficult.

Packing problems can be overcome by in-situ synthesis of monolithic stationary phases, starting from liquid precursors. Moreover, retaining frits are not required as the monolithic scaffold can be covalently attached to the wall, which makes the system likely also more robust than packed microchannels [79]. The wide variety of monomers available allows to tune selectivity as desired. Alternatively, reactive monomers can be incorporated that are prone to functionalization afterwards and (local) grafting approaches can be pursued to create the desired surface chemistry after optimizing the porous properties of a ‘generic’ chromatographic support structure [80,81]. An alternative of the above mentioned approach for the functionalization of monolithic supports containing the desirable reactive moieties can be the use of click chemistry reactions i.e. copper catalyzed azide cycloaddition, thio-ene/-yne reaction and Diels-Alder reaction [82–84]. Figure 4.6 shows a polymer monolithic support structure in the confines of an interconnected microchannel structure of a spatial 2D-LC chip [35]. In the chip displayed in 4.6A, the polymer monolith was synthesized in all the microchannels, i.e., in the 1D channel, in the parallel 2D channels, and in the flow distributor. Fig 4.6B shows the presence of the polymer monolithic support structure only in the 2D channels, with Fig. 4.6C a zoom-in displaying the globular structure of the polymer monolith. By using photoinitiation of the polymerization reaction and applying a photomask covering the 1D channel and flow distributor...
monolith synthesis could be confined to the desired location. Themelis et al. reported the in-situ synthesis of monolithic support structures in the microchannels of a spatial 2D-LC chip integrating the active-valving concept to confine the flow during the 1D stage, see Chapter 5 for the detailed discussion (Fig. 4.6D) [37]. After sample transfer, sample focusing was achieved at the inlet of the parallel 2D channels filled with monolith (see Fig. 4.6D-E) which was followed by a gradient RPLC separation. Passamonti et al. described the confinement of a polymer monolith inside a titanium device with controllable hot and cold regions [85]. Monolithic structures were created in capillaries and inserted into the channels of a device that was in direct contact with Peltier elements for cooling and heating. Also, a titanium device was developed that integrated a heating jacked (maintained at 70°C) and cooling jacked (maintained at 10°C), allowing to locally synthesize a monolith structure in the region of the hot zone. Excellent review papers describing the incorporation of different types of stationary phases in microfluidic devices have been published by Kutter [86], Grinias and Kennedy [87], and Yuan et al. [88].

Figure 4.6. Microfluidic devices for comprehensive spatial 2D-LC integrating polymer monolithic support structures in the microchannel. In (A) monolith is present in the 2D flow distributor, and in the 1D channel and 2D parallel microchannels. In (B) a photomask was applied during photo-initiation and monoliths are only present in the 2D channels. In (C) a magnification of the interconnected globules is displayed. (D) shows a spatial chip that integrates the active-valving concept with a polymer monolith present in the 2D flow distributor and parallel 2D channels. (E) is a cross section of the chip displayed in (D) showing monolith present only in the target regions and not in the 1D channel, with F displaying a zoom-in of the monolith in the microchannel displayed in (E). Fig. 4.6A-C taken from [35]. Fig. 4.6D-F taken from [37].
4.3.4. Possible detection approaches

The detection options for spatial 3D-LC can be divided into two main categories depending on the elution mode used during the third development stage, i.e., a space-based ('LC) or time-based development ('LC). In a spatial 3D-LC system with a space-based 3D ('LC × 'LC × 'LC), the sample never leaves the separation body and needs to be detected in-situ. In such case, confocal microscopy can be applied to either scan parts of the 3D body (targeted detection) or scan the 3D body to get a comprehensive overview of all constituents present in the sample mixture. Mathies et al. filed a patent request for a laser excited confocal microscope fluorescence scanner allowing to detect sample components within a slab gel after electrophoresis [89]. The proposed device was set up in such a way to have an estimated depth of view at the order of micrometers. Targeted confocal spectroscopy can be pursued with high detection sensitivity, and is a viable option for biomarker validation applications, however a conventional 2D-LC or 3D-LC heart-cutting approach using coupled columns is likely more straightforward. The depth of penetration underneath the surface of the studied object depends on the opacity of the material and the working distance of the objective lens. This limits the working depth (Z direction) to some millimeters at the most which consequently limits the 3D length [90]. Also, one needs to consider refractive index effects induced by the presence of stationary phases in the 3D body, which leads to various scattering effects compromising the detection quality [91]. The resulting chromatogram could have similar format as that developed by Jorgenson et al., as described in Section 4.1.

When applying a time-based separation during the 3D development stage ('LC × 'LC × 'LC), analytes are eluting out of the separation body. In this case, detection can be established either at the chip interface at multiple points simultaneously at the chip outlets (equal to the number of 3D microchannels) or the effluent can be collected for subsequent offline analysis. A promising approach that may be implemented for spatial 3D-LC was developed by Liu et al. this group reported on capillary array electrophoresis where up to 128 capillaries could be detected in parallel using optical detection [92]. A rotating mirror with 18 rotations per second was utilized to direct emitting and reflecting light yielding a scan rate of ~ 16 Hz per capillary and enabling semi-parallel detection. Alternatively, Fan et al. developed a microfluidic device with parallel and high-throughput multicolor fluorescence detection in 32 microchannels [93]. A measuring platform allowing to establish simultaneous fluorescent detection of droplets travelling through 64 parallel channels was developed by Schonbrun et al. [94]. This set-up of microfluidics and optics utilizing a high-speed CMOS camera allowed measuring the
fluorescence from 180,000 droplets per second. Another possibility could be the use of in-chip integrated waveguide arrays that would guide fluorescent beams from an external source on the 3D channels array in a design adapted from the one of Morgensen et al. [95]. The emission energy would be collected by an array of photomultipliers placed next to each 3D channel. Nevertheless, to apply optical detection as described here, the microdevice needs to be optically transparent to allow for penetration of the absorbed or emitted electromagnetic radiation.

Hyphenation of spatial multi-dimensional LC systems to mass spectrometric (MS) detection is mandatory for proteomics analysis. When considering ESI interfacing no straightforward solutions exist yet, allowing to hyphenate spatial 3D-LC to MS. One approach that may become of interest is the development of multiplexed electrospray ion sources allowing to sample from parallel channels. Four-channel multiplexed ESI sources are commercially available and intersprayer cross talk is low (< 0.08% at concentrations of metabolites as high as 1000 ng/mL) [96]. The current-state-of-the-art multiplexed electrospray ion sources allow to sample up to eight different nozzles [97]. Using microlithographic fabrication techniques, a miniaturized array of nozzles could be developed with a density of 250 ESI spray sources/cm² [98].

Many groups have been working on miniaturizing certain components of mass analyzers, which was greatly aided by the high precision sub-µm structuring capabilities of photolithographic and etching techniques utilized to construct microelectromechanical systems (MEMS) [99,100]. The need for portable and field-deployable mass analyzers has resulted in the miniaturization of different types of mass filters, including time-of-flight analyzers [101,102], quadrupole mass filters [103,104], and ion traps [105–109]. Ion trap MS instruments may be ideally suited for hyphenation to microfluidic chip devices, due to their straightforward design and associated low-power consumption which comes with a miniaturized ion-trap design. However, miniaturization also diminishes the trapping potential well depth, resulting in a degradation of mass resolution. To tackle this issue, an array of cylindrical ion traps has been integrated on a single device by Cooks [99,110] and Ramsey [106,107]. More recently, Fico et al. demonstrated the potential of producing parallel linear ion traps using 3D-printing stereolithography [105]. This technology possibly holds great potential for hyphenation of miniaturized MS detection to spatial 3D-LC devices.

A more straightforward strategy for interfacing spatial 3D-LC to mass spectrometry may be to detect analytes after immobilizing the effluent from the final separation on a suitable substrate at regular intervals followed by offline mass spectrometry imaging (MSI) [111]. This concept would be similar to what has been done for many years now in the field of tissue
imaging, where an immobilized tissue section is coated with matrix and the sample is measured in a raster process yielding spatially resolved mass spectra [112,113]. The acquired MS data-sets present high dimensionality not only due to the fact that consecutive arrays of deposited effluent will be scanned by the laser (representing different points of the bidimensional plane as it develops in the 3D) but also because different selected m/z ions can be displayed, originating from a single raster process [113]. Regarding the interfacing, several studies have been conducted describing the possibilities of capillaries [114] and microfluidics coupled with off-chip MSI analysis [115]. Tsao et al. reported on the development of interfacing microfluidics with MS using a robotic spotting system, see Fig. 4.7A for the experimental set-up [116]. A thermoplastic microfluidic chip containing eight parallel channels was coated with Teflon at the channel exits to increase the hydrophobicity, encouraging droplet formation, see Fig. 4.7B. This multiplexed chip was placed on a X, Y, Z stage, which was positioned 2 mm away from a laser-desorption-ionization (LDI-) MS target plate. Stepping motors were utilized to mechanical deposit effluent from the microchannel outlets on the target plate, yielding spot volumes of 104 to 190 nL, see Fig. 4.7C. Tsao et al. reported on a multichannel piezoelectric dispenser integrating picoliter dispensing tips to deposit arrays of solvent nanodroplets onto tissues prior to ambient liquid extraction MS [117]. Alternative approaches used to deposit sample volumes on target plates include also electrospray and piezo-electrospray deposition [118–120], use of a pressure-pulse dispenser [121], and acoustic spotting [122]. As droplets are easily accessible, assay reagents or matrix molecules can be added to all or selected droplets only [123]. Different ionization techniques are compatible with MSI interfaces, including LDI [124], desorption electrospray ionization [125], and ambient liquid extraction MS [126]. A high-throughput MALDI-MS detection stage can be used to realize ultra-high throughput MS screening following spatial 3D-LC separations [17]. This instrument uses a rotating sample stage and a 10 kHz laser repetition to achieve high pixel rates up to 40 pixels/second. This technology has already demonstrated its merit for advancing diagnosis and patient care, enabling rapid analysis of frozen tissue sections which greatly improves clinical decision making [126]. The automated solution can process a 1536-well plate under 8 minutes, which was demonstrated for drug development by analyzing over one million drug candidates in one week [127].
**Figure 4.7.** Interfacing of a microfluidic chip containing 8 parallel channels to laser-desorption-ionization mass spectrometry by automatic robotic spotting to mechanically deposit effluent on the target plate. (A) shows a photo of the experimental set-up, (B) shows the effect of a hydrophobic coating at the channel exit on droplet formation, and (C) depicts the result after sequentially depositing effluent on the target plate. Taken from [116].

### 4.4. Concluding remarks

A spatial 3D-LC device has unarguably very high potential to offer in the field of separation sciences and particularly in modern proteomics and biomarker discovery. The initial step of prototyping devices able to withstand a few hundreds of bars has already been demonstrated via different manufacturing methods, yet major challenges must be addressed before obtaining a fully and properly functional spatial 3D-LC device. One of the most important prerequisites towards a fully operative device is the high-pressure flow confinement between the subsequent dimension developments. This has been recently studied and proven at the interface between the 1D and 2D but still needs to be implemented at the interface between 2D and 3D where only computational works are present in the literature. Another fundamental step that must be accomplished is the incorporation of different and orthogonal separation mechanisms of both pressure and electro-driven nature in each dimension of the device. Until now the use of organic monoliths has been promoted as the most feasible way of integrating stationary phases in such devices as packing of multichannel thermoplastic chips can be rather challenging, though not impossible. The last and crucial step towards a proof-of-concept spatial 3D-LC separation is
coupling the device with a detection system. As spatial 3D-LC is still being developed, a separation example is not yet available. Considering the importance of MS technology in the proteomics research field, the most realistic approach for establishing detection is by depositing the effluent of the third dimension on a MALDI plate enabling offline MS detection. Technical issues related to 2D imaging data alignment and 3D visualization have already been identified and to a large extent addressed. For example, Xiong et al. explored different methods to reconstruct a 3D data set retaining mass spectral information for 3D-MS imaging [128]. Different aspects, including data reduction, tissue section alignment, data visualization, as well as statistical analysis using PCA and cluster analysis were considered, which are also applicable for the construction and analysis of data in spatial 3D-LC. An excellent review paper was also published by the Caprioli research group in 2013 describing critical steps in the 3D image visualization [113]. Different software packages exist to reconstruct these images and inspect them from different angles allowing to assess the spatial distribution of molecules in the sample. Future research in the detection part might also include parallel miniaturized mass filters on the device for sensitive online MS data acquisition. Finally, method development in spatial 3D-LC devices will become even more challenging and computational methods should be used in the future to simplify the life of the chromatographer towards achieving an optimized separation.

4.5. References


Towards Spatial Comprehensive Three-Dimensional Liquid Chromatography


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TOWARDS SPATIAL COMPREHENSIVE THREE-DIMENSIONAL LIQUID CHROMATOGRAPHY


CHAPTER 5

ENGINEERING SOLUTIONS FOR FLOW CONTROL IN MICROFLUIDIC DEVICES FOR SPATIAL MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY

Chapter based on the article:

*Engineering solutions for flow control in microfluidic devices for spatial multi-dimensional liquid chromatography*

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5.1. Introduction

Mass spectrometry (MS)-based proteomics has become an indispensable tool in biomarker discovery and validation studies [1–3]. It has been estimated that the human cellular proteome contains a cumulative copy number of $10^9$-$10^{11}$ protein molecules per cell [4]. To reduce the spectral complexity of contemporary life-science mixtures and at the same time prevent ion-suppression effects induced by co-elution, highly efficient separation methods are required prior to MS infusion [5]. The introduction of ultra-high-pressure liquid chromatography (UHPLC) combined with (long) columns packed with sub-2-micrometer particle technology has led to a significant gain in resolving power [6]. However, the peak capacity provided by one-dimensional UHPLC technology, even when utilizing long (shallow) solvent gradients, is not sufficient to account for hundreds of thousands of different protein isoforms [7–9]. Conventional column-based two-dimensional liquid chromatography (2D-LC) has the potential to reach very high peak capacities [10], when utilizing orthogonal developments [11], and its applicability in proteomics research has been readily demonstrated. A disadvantage is the sequential analysis of fractions sampled from the first-dimension development, leading to relatively long analysis times, making the technology intrinsically unsuitable for biomarker validation studies. Spatial three-dimensional liquid chromatography (3D-LC) constitutes a novel separation concept, in which analytes are separated by their position in a three-dimensional space characterized by (X,Y,Z) coordinates [12]. By utilizing orthogonal developments, very high resolving power (potentially even higher than 50000) can be reached since the maximum peak capacity is the product of the three individual dimensions [11,13]. In contrast to conventional multi-dimensional approaches where fractions are analyzed sequentially, spatial LC enables the develop of all fractions in parallel. This can result in unprecedented performance in terms of peak-capacity-per-unit-time, i.e. the peak production rate, allowing for biomarker discovery and validation studies.

The first prototype microfluidic device for spatial 3D-LC, integrating over 270 interconnected microchannels, was demonstrated in 2015 [12]. However, technology development for spatial multi-dimensional separations performed at high-pressure is still in its infancy. To generate a homogenous flow across parallel (2D) channels, flow distributors were developed based on prototypes for 1D-LC by Regnier et al.[14] and Desmet et al. [15]. Based on insights obtained with computational fluid dynamics simulations and executing on-chip flow experiments, fractal bifurcating flow distributor designs were optimized [16,17]. Also, a fractal 3D flow distributor was developed to distribute a flow across 256 parallel channels.
filling the 3D space [18]. A critical aspect in establishing spatial separations is to restrict the subsequent developments within their predefined spatial domains, to effectively utilize all the available separation space [19,20]. A variety of different approaches, including pneumatic [21,22], electrokinetic [23–25], magnetic actuators [26], and ‘smart’ polymer materials [27,28], that can undergo phase changes have been explored to confine and block flows in microchannels. The current state-of-the-art microvalves are characterized by rapid response time down to milliseconds. An alternative approach was presented by the Whitesides’ research group, who explored a torque actuated “TWIST” valve, where screws were used to collapse the microchannel fabricated in a poly(dimethylsiloxane) top substrate when pressed on the bottom glass substrate [29]. Guler et al. developed a plug microvalve containing a cylindrical through-hole that can be aligned with microchannels via rotation, yielding a maximum pressure resistance of 2 kPa [30]. Unfortunately, many concepts are incompatible with the pressure resistance requirements of HPLC (5–40 MPa) or even higher. Furthermore, parallelization of the actuator operation to close and open parallel microchannels or a section of the microfluidic device is an important aspect. Gong et al. reported a 3D printed multiplexer concept [31], integrating 5 parallel microvalves on-chip that can be actuated with a 15 ms phase interval and withstanding pressures up to 70 kPa. Recently, the use of freeze-thaw valves with switching times between 1 and 2.5 min was proposed by Nawada et al. allowing to withstand up to 20 MPa of pressure [32].

In this study, we explore different solutions to control and confine flow on a microdevice built for multi-dimensional spatial liquid chromatography. First the channel design was optimized, with respect to flow distribution across parallel second dimension (2D) channels. The performance of bifurcating flow distributors with a different channel layout was experimentally assessed, applying flow rates in a wide range from 50 to 1000 µL/min (at the distributor inlet). To confine the flow during the first-dimension (1D) development, the potential of using physical barriers was assessed. Microchannels with reduced channel cross section were integrated on-chip and the effects of implementing porous polymer-monolithic substrates to further enhance flow restriction were experimentally investigated. Finally, the development of an active-valving concept is presented providing a leak-free approach to confine developments at predetermined location.
5.2. Experimental

5.2.1. Reagents and materials

Butyl methacrylate (BMA, 99%), ethylene dimethacrylate (EDMA, 98%), 1-decanol (99%), cyclohexanol (99.9%), 2,2 dimethoxy-2-phenylacetophenone (DMPA, 99%) ethylene glycol diacrylate (EGDA, 90%), methyl methacrylate (MMA, 99%), benzophenone (BP, reagent plus, 99%), cyclohexane (anhydrous, 99.5%), sudan black B pigment, sudan I (dye content ≥ 95%), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), dimethylsulfoxide (DMSO), sodium carbonate anhydrous (99.9%), sodium bicarbonate anhydrous (99.9%), and phosphate buffer saline (PBS) were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands). Monomers were purified by flushing the liquids through a column packed with activated basic alumina (VWR, Leuven, Belgium). 2-propanol (IPA, technical grade) was purchased from VWR. Dichloromethane (99.9%) and acetonitrile (ACN, HPLC supra gradient) were purchased from Biosolve (Valkenswaard, The Netherlands).

5.2.2. Chip fabrication

Fig. 5.1. depicts two designs of microfluidic devices for spatial 2D-LC, integrating physical barriers (Fig. 5.1A) and the optimized active-valve approach (Fig. 5.1B), respectively. The microdevices were constructed from cyclic olefin copolymer (COC) substrate (TOPAS advanced polymers, grade 8007, Kunststoff-Zentrum, Leipzig, Germany) using a computer-numerical-control micro-milling machine (Datron M7 Compact, Mühltal-Traisa, Germany). Prior to milling the microchannels, a face milling step was applied to reduce the surface roughness. The microchannels were constructed in both the top and bottom chip substrate by using a 200 μm diameter single-flut endmill and a 250 μm diameter ball-nose milling tool. For the device in Fig. 1A the 1D channel length was 35 mm while for the active valving device (Fig. 1B) an 80 mm long polyether ether ketone (PEEK) tube with 500 μm i.d. was threaded inside a perfluoroalkoxy alkane tube (PFA, Postnova Analytics, Landsberg am Lech, Germany), containing eight uniformly spaced and aligned 500 μm i.d. through-holes. Both devices had 2D channel lengths of 35 mm. In both chip designs, 400 μm i.d. through-holes were drilled in the bottom plate serving as 2D flow in and outlet. An irreversible bond between top and bottom chip substrates was obtained via solvent-vapor-assisted bonding [33]. After exposing the top substrate to cyclohexane vapor, bottom and top substrates were aligned with
a precision of 2 µm using an in-house, custom-made, aluminum holder, and pressed together using a hydraulic press for 45 min.

**Figure 5.1.** Schematic representation of two different layouts of a microfluidic device for spatial 2D-LC. (A) shows a chip design utilizing physical barriers using cross-section constrictions (200 µm i.d. channels marked in red) in the flow distributor and the column head of the 2D channels. (B) a device equipped with a rotating active-valving system consisting of a PEEK tube threaded in a PFA sleeve, both containing 8 drilled through-holes. During the 1D development the through-holes are closed, during 2D development the through-holes are aligned with the 2D flow distributor and 2D channels. The black colored lines represent 500 µm i.d. channels and the green dashed line marks the effective separation space for the 1D channel.

Precision-machined bottom parts for a holder to accommodate the chip device were constructed from aluminum, featuring height-tunable side clamping blocks as well as flat-bottom nanoport connections (Upchurch Scientific, Oak Harbor, USA) compatible with 360 µm o.d. capillary fused-silica tubing. Top parts for the holder were milled from transparent PMMA and bolted together with controlled torque.
5.2.3. Integration of polymer-monolithic substrates

To enable a covalent attachment of the monolithic substrates to the COC channel wall, a surface pretreatment was performed. Therefore, microchannels filled with a mixture consisting of 50 wt% MMA, 50 wt% EGDA, and BP (20 wt% with respect to the total monomer content) were irradiated with UV light at $\lambda = 254$ nm for 25 min (XL1000/F, Spectroline, USA), and flushed with ACN using an HPLC pump (LC Packings, Amsterdam, The Netherlands). Next, methacrylate-ester-based polymer monoliths were synthesized \textit{in-situ} in the microchannels applying a polymerization mixture consisting of 24 wt% BMA, 16 wt% EDMA, 54 wt% 1-decanol, 6 wt% cyclohexanol, and DMPA (3 wt% with respect to the total monomers). To restrict the polymerization at the desired location while applying UV light ($\lambda = 365$ nm for 5 min), polyoxymethylene photomasks were constructed using micromilling. Afterwards, the channels were flushed with ACN.

5.2.4. Flow-control experiments with on-chip optical/fluorescence detection

BSA was labelled using 1 mg/mL FITC dissolved in anhydrous DMSO. A 2 mg/mL BSA dissolved in sodium carbonate buffer solution (0.1 M at pH = 9) was prepared. For every 1 mL of protein solution, 50 $\mu$L of FITC solution was added in small aliquots under continuous stirring and the reaction was incubated in the dark for 8 hours at 4°C. Next, ammonium chloride was added reaching a final concentration of 50 mM and the reaction was incubated for another 2 hours at 4°C. Excess of FITC was removed applying a NAP-25 gel filtration column and the protein adduct was recovered in PBS buffer. The on-chip monitoring of dyes and the FITC labelled protein flows was achieved with a digital optical/fluorescent microscope (Dyno-Lite, Naarden, The Netherlands).

Prior to sample loading, the monolithic stationary phase situated in the \textsuperscript{2}D channels was equilibrated by flushing several column volumes of a 5:95% (v/v) ACN:H$_2$O and the \textsuperscript{2}D inlet and outlets were closed by means of an external 6 port-2 position valve. Next, fluorescent labelled protein dissolved in aqueous PBS buffer was introduced in the \textsuperscript{1}D inlet until reaching the channel outlet. In order to reassure a non-biased flow confinement test, the fluorescent tagged analyte was continuously injected until it reached the waste outlet of the system through the valve. After blocking off the \textsuperscript{1}D channel, the on-chip valving mechanism was rotated 90° aligning the through-holes with the \textsuperscript{2}D channels, and analyte focusing of a FITC labelled
protein at the channel inlets was achieved applying 5:95% (v/v) ACN:H₂O as the mobile phase at a flow rate of 100 µL/min.

5.3. Results and discussion

5.3.1. Chip design and advancing the channel layout

To realize multi-dimensional spatial LC separations, we have opted for a microfluidic chip design featuring parallel 2D channels. As the chip is composed of different substrate layers, the parallel channel design provides enough contact area to effectively bond the chip substrates, yielding a device compatible with operating pressures over 10 MPa. As efficient packing of complex channel structures is extremely difficult to accomplish, implementation of monolithic substrates in microchannels starting from liquid precursors is more attractive. Since spanning of cross sections larger than 1 mm frequently resulted in rupture, we opted to integrate 500 µm i.d. 2D separation microchannels.

To effectively operate 500 µm i.d. (individual) separation channels containing a polymer-based monolithic stationary phase for gradient analysis, flow rates 10 up to 40 µL/min are recommended (depending on the macropore and globule diameter) [34], which corresponds to a flow rate at the 2D flow distributor inlet of 80 up to 320 µL/min, using the chip design with 8 parallel 2D channels, depicted in Fig. 5.1A. The performance of a flow distributor with a 175° angle between the bifurcations (previously studied based on computational fluid dynamics modelling [16]) was assessed applying a broad flow rate range between 1000 µL/min down to 50 µL/min. Fig. 5.2A shows that the outer branches on each bifurcation level (marked with an asterisk) are less permeated than the respective inner channels, leading to an inhomogeneous flow profile. The inhomogeneity in flow distribution is more pronounced at lower flow rate, see Supplementary video 1 (see video in [35]). When utilizing a flow distributor with a 180° angle between the bifurcations, a nearly perfect homogenous flow profile is achieved across all parallel channels, independent of the flow rate applied, see Supplementary video 2 (see video in [35]).
Figure 5.2. Performance assessment of 2D flow distributors containing three levels of bifurcations and an angle between the branches of (A) 175° and (B) 180°, respectively. The inlet flow rate was 100 µL/min, resulting in a volumetric flow rate of 12.5 µL/min in each individual 2D channel with a cross section of 500 µm. The red dotted line provides a guide for the eye highlighting the differences in flow velocities in the different 2D channels.

A spatial 2D-LC chip based on the design in Fig. 5.1A was developed, integrating the optimized bifurcating 2D flow distributor with three levels of bifurcations featuring an angle of 180°, a 500 µm i.d. circular channel for the 1D development, eight 500 µm i.d. parallel 2D channels (circular cross-section), and a flow collector. In an attempt to minimize leak flow during the 1D development into the 2D flow distributor and 2D channels, external valves were used, allowing to introduce dye in the 1D channel meanwhile blocking off the in- and outlets of the 2D flow distributor and collector. In addition, the cross section of the flow distributor and the 2D channel inlets was reduced from 500 to 200 µm. During the 1D development significant leak flow was observed in the microchannels of the flow distributor and in the 2D separation channels, see Fig. 5.3A. To further decrease the permeability of the chip segments confining the flow during the 1D development, polymer-monolithic substrates were synthesized locally in the microchannels positioned vertically with respect to the 1D channel by utilizing photomasks, leaving only the 1D empty without stationary phase (see Fig. 5.4). Fig. 5.3B and C show screenshots obtained during and directly after filling of the 1D channel, respectively. Even at low pressure operation (0.7 MPa at the 1D inlet, open 1D outlet, and closed 2D in- and outlets) some leak flow was observed towards the 2D microchannels and flow distributor channels positioned closer to the 1D inlet, although to a much lesser extent compared
to Fig. 5.3A. Fig. 5.3D shows sampling of the ¹D effluent and elution in the parallel ²D channels.

Figure 5.3. Screenshots of microfluidic device for spatial 2D-LC utilizing physical barriers, *i.e.*, the cross sections of microchannels situated at the ²D flow distributor and the ¹D channel inlet are reduced from 500 µm to 200 µm i.d. (A) During filling of ¹D channel (via bottom inlet) the ²D inlet and outlets are blocked off via external valves but significant leak flow is observed. In (B-D), monolithic support structures have also been synthesized in the ²D flow distributor and ²D channels, decreasing the permeability. (B) and (C) shows the ¹D flow confinement during filling and only 20 s after filling the ¹D channel with dye, respectively. (D) shows elution profiles of the ¹D content transferred into the ²D channels applying at a flow rate of 100 µL/min at the ²D flow distributor inlet (5 seconds after start ²D development).

Figure 5.4. Photograph of a chip for spatial 2D-LC (based on the schematic depicted in Fig. 4.1A) that contains cross section constrictions and localized polymer monoliths in the ²D microchannels and flow distributor as means of flow confinement.
Note the highly homogenous flow profile in the 2D channels. However, in the current design, recovery problems (when sampling from the 1D channel to the 2D channels) are observed due to the limited number of 2D channels.

When considering spatial multi-dimensional separations, the first-dimension LC mode could also utilize a chromatographic support structure, thus the permeability differences between 1D and 2D will be less pronounced. This will inevitably lead to more enhanced leak flow, compromising the available separation space and, hence, minimizing the maximum achievable peak capacity. Based on this study, the bifurcated flow distributor with 180° angle and 500 µm parallel channel design were kept.

5.3.2. On-chip active valving

To advance flow confinement and prevent adsorption of analytes at the interface between the 1D and 2D development an on-chip active valving approach was pursued. This concept (see Fig. 5.1B) is based on a rotating axis, comprised of a PEEK tube (that constitutes the 1D separation channel), containing through-holes that either can be closed when these are positioned perpendicular with respect to the 2D plane or open when aligned with the 2D flow distributor and the 2D channels after rotation of the axis by 90°. Note that this prototype, in contrast to the device discussed in “Section 5.3.1”, does not incorporate physical barriers by reducing the cross-section diameter of the channels. A first demonstration of this concept was developed by applying solvent-assisted drilling to introduce a PEEK tube in a microfluidic device from poly(methyl)methacrylate, allowing to confine the 1D development.

Figure 5.5. Prototype of a microfluidic device containing a rotating PEEK tube as means of flow confinement.
After drilling a small i.d. channel the PEEK tube was mounted in the drilling machine. Dichloromethane was sparsely added on the chip while the PEEK tube was drilled at low speeds into the existing channel, creating a tight fit between chip and tube. After evaporation of the dichloromethane a tight fit was obtained allowing to rotate the PEEK tube using a gripper, but not by hand, see Fig. 5.5 for a photograph of the prototype chip device. Although flow confinement was achieved, minor leakages from the 1D channel to the 2D channels appeared after repeated switches. A second-generation prototype was developed using COC as substrate and micromilling a support groove for the PEEK tube, see Fig. 5.6 [36]. The groove exactly matched the 500 µm i.d. × 1.58 mm o.d. PEEK tube. Furthermore, the chip substrate plates were bonded with the PEEK tube inserted ensuring alignment of the through-holes with the flow distributor and the 2D channels. Fig. 5.6A and B shows photo captures during the 1D development. Progressive filling of the tube is noticed by the appearance of black dye in subsequent through-holes perforating the PEEK tube. However, significant leakage was observed at low pressure operation (0.15 MPa).

![Figure 5.6.](image)

**Figure 5.6.** Second prototype of a microfluidic device containing a rotating PEEK tube as means of flow confinement. (A) Progressive filling of the channel is documented until the through-hole marked with the red asterisk. (B) Screenshot capture of the device 5 s after completely filling the 2D channels, demonstrating significant leak flow in the flow distributor and 2D channels.

Adamopoulou *et al.* recently reported on a similar concept and corresponding leakages at the channel inlet and interface [37]. To realize a leak-free device, the PEEK tube was inserted in a PFA sleeve (both containing through-holes), prior to solvent-vapor assisted bonding of the chip substrates, see Fig. 5.1B for the optimized chip design. A photograph of the device is depicted in Fig. 5.7A. The PFA sleeve is characterized by a high elasticity and can thus be compressed.
by the vertical forces applied via a custom-made encasing holder bolted using controlled torque and pressure applied via a pneumatically actuated piston. During the device’s operation the pressure was instantaneously released in order to rotate the valve and then applied again to ensure leak free operation. Successful operation of the active valving is demonstrated in Fig. 5.8A-B and Supplementary videos 3 and 4 (see videos in [35]), using dye and optical detection and FITC in combination with on-chip fluorescence detection.

**Figure 5.7.** (A) Photograph of a spatial 2D-LC chip implemented with the active-valving concept and monolithic support structures at the flow distributor and 2D channels and (B-E) scanning electron microscopy images of cross and interface sections of the chip. (F) shows a zoom-in SEM image of the monolithic interconnected structure synthesized *in-situ* in the separation channel.

**Figure 5.8.** Leak-free operation of active-valving concept using on-chip fluorescent detection: (A) shows confinement after 1D injection and development applying a flow rate 100 μL/min. (B-D) shows 2D development:
(B) is without stationary focusing yielding broad 2D band, (C-D) is achieved with stationary-phase focusing of labelled protein captured at the 2D channel inlet remobilization using a step gradient.

After confining the fluorescent marker solution in the 1D separation channel during the first-dimension development, the rotation of the axis is executed and FITC is effectively transferred by flow entering from the 2D flow distributor, feeding the parallel 2D channels while maintaining a relatively homogenous flow profile. No leak flow was observed during the first-dimension development applying pressures as high as 5 MPa, measured by using a restrictor at the 1D channel outlet, nor during the 2D development. Differences in flow velocities in the parallel 2D channels are likely caused by a slight off-set in injection time due to elasticity of the PEEK during rotation while applying a high torque via the holder to prevent leakages, see Supplementary video 3 (see video in [35]). To univocally prove that flow was introduced in the chip via the flow distributor during the 2D development, a 1 µL loop filled with fluorescent tracer was switched in-line with the mobile the mobile phase supply when recording Supplementary video 4 (see video in [35]).

5.3.3. Stationary-phase focusing and chromatographic separation

Stationary-phase focusing of analytes, due the strong interaction of analytes with the stationary phase when applying aqueous elution conditions (in RPLC mode), between subsequent chromatographic developments (reducing the peak volumes) is highly desirable to counteract chromatographic dilution. When the tail of a peak experiences a mobile phase that is a stronger eluent (ACN-rich mobile phase), compared to the one at the front of the same band, the tail of the peak moves faster and a peak-focusing effect is observed. This methodology opens the possibility for scaling of channel dimensions such as to achieve high mass and volume loadability during the first-dimension separation using a large i.d. channel, and downscaling microchannels in subsequent 2D development to enhance the flow rate compatibility with for example mass-spectrometry detection, required for proteomics applications. Fig. 5.7 depicts the chip prototype and the zoom-in panels display a cross section of the microfluidic device highlighting the tight fit between the COC chip body and PFA sleeve as well as between PFA sleeve and PEEK tube, resulting in leak-free high-pressure operation. Applying a UV photo-initiated free-radical polymerization and a photomask, monolithic stationary phases were locally created in-situ, in the flow distributor and collector, in the through-holes of the PFA sleeve, and in the 2D separation channels displaying straight and
well-defined interfaces, while no monolith is present in the PEEK tube that lacks UV transparency. The advantage of integrating the monolithic substrate in the flow distributor is that it reduces the gradient dwell volume. The first proof-of-concept of combining active-valving technology with stationary-phase focusing is demonstrated for a fluorescent labeled protein in Fig. 5.8C and D. BSA labelled with FITC dissolved in aqueous sodium carbonate buffer was introduced in the 1D channel and confined in the 1D channel via active valving, see Fig. 5.8A. After the 1D development, the axis was rotated and effective focusing of BSA was achieved at the 2D channel inlets applying aqueous elution conditions introduced via the 2D flow distributor at a flow rate of 100 µL/min, see Supplementary video 5 (see video in [35]). When introducing an ACN-rich (95%) mobile phase, the protein was remobilized while compressing the chromatographic band, and eluted towards the channel outlet, see Fig. 5.8D. Differences in flow velocities and peak widths observed in the different 2D channels may be induced by inhomogeneity of the monolithic stationary phase or manual imprecisions when turning the rotating valve in the open position. Quantitative data on channel-to-channel (n = 8) and chip-to-chip (n = 4) repeatability addressing mobile-phase velocity, band width, and intensity measured at the apex for fluorescent labelled albumin eluting during the 2D dimension development are provided in the Fig. 5.9. Fig 5.9A shows the peak width obtained during the focusing step just before remobilization takes place, when applying a solvent step gradient. Note that the smaller the band width, the better the stationary-phase focusing is. Fig 5.9B shows the difference in fluorescence intensity measured at the apex. The different bars represent the performance measured for 4 different chips. The red error margins represent run-to-run standard deviation (SD) values for 3 consecutive measurements. Finally, Fig. 5.9C shows the repeatability in linear mobile-phase velocity measured at the moment of elution after applying a mobile phase step gradient. The error bars represent chip-to-chip SD for n =3.

**Figure 5.9.** Channel-to-channel, run-to-run (n = 3), and chip-to-chip (n = 4) repeatability data on peak width (A) and fluorescence intensity measured at the peak apex (B) during stationary phase focusing applying an aqueous mobile phase. (C) Presents the differences in linear mobile-phase velocity at the moment of elution after applying the solvent step gradient. The error bars represent the chip to chip standard deviation for 4 chips (A and B) and 3 chips (C), respectively.
The first demonstration of active valving for flow confinement of a mixture of dyes during the 1D development, followed by stationary-phase focusing at the 2D channel inlets and a subsequent chromatographic separation is displayed in Fig. 5.10.

Figure 5.10. Demonstration of stationary-phase analyte focusing (A) and subsequent on-chip chromatographic separation applying gradient reversed-phase chromatography (B-D) after 1D flow confinement of the sample via active valving. The sample mixture was composed of sudan black B and sudan I. Focusing of the dyes was obtained by introducing 5:95% (v/v) ACN:H₂O at 100 µL/min for 1 min via the flow distributor, followed by the separation when introducing a linear aqueous-ACN gradient from 5% to 90% for 10 min. The chromatograms presented in (C) and (D) were extracted from channel six counting from the left recorded 4.2 min and 5.6 min, after the gradient start, respectively.

After active valving, a mobile phase composed of 5:95% (v/v) ACN:H₂O was introduced at a flow rate of 100 µL/min for 1 min via the 2D flow distributor allowing to focus the dye components in a narrow band at the inlet of the 2D channels filled with monolithic support structure, see Fig. 5.10A. Subsequently, a 10 min linear gradient reaching 90% ACN was applied, allowing to resolve the mixture components with baseline resolution. Fig. 5.10B presents a screenshot of the chip showing blue (more retained) and yellow bands, corresponding to sudan black B and sudan I respectively, eluting during the gradient. After travelling only 10 mm, baseline resolution was achieved between the dyes, as seen in the
extracted chromatograms from channel 6 (counted from the left) displayed in Fig. 5.10C. The data recorded via the optical microscope were converted and reconstructed in chromatograms using an in-house made MATLAB script. As expected, by increasing the travelled distance the resolution is enhanced, see Fig 5.10D, as the incremental distance ($\Delta t_R$) increases more rapidly than the peak width.

5.4. Concluding remarks

The design of flow distributors of microfluidic devices for spatial multi-dimensional separations has been improved and different approaches for the realization of flow confinement were explored. Integrating physical barriers on-chip to confine flow during $^1$D, $^2$D (and possibly $^3$D) developments may work to some extent, but more likely compromises separation space and hence chromatographic performance. In contrast, an active-valving approach has been successfully developed to confine the flow during $^1$D developments in a spatial 2D-LC chip and applied in combination with stationary-phase focusing between the developments. The active-valving approach allows to fully utilize the separation space provided by the chip, maximizing the potential peak capacity. To tackle the recovery problem, the number of $^2$D channels should be greatly increased. The stationary-phases focusing/remobilization concept is critical towards the operation of a spatial chip for spatial multi-dimensional LC since this counteracts chromatographic dilution. Furthermore, the stationary-phase focusing concept allows to tune the dimensions of the microfluidic channels in each dimension, i.e., having a relatively large i.d. $^1$D channel, maximizing the sample loadability and establishing small i.d. $^2$D channels to operate at low volumetric flow rates. This allows to hyphenate spatial multi-dimensional chip devices to mass-spectrometry imaging detection.

5.5. References


[23] G. V. Kaigala, V.N. Hoang, C.J. Backhouse, Electrically controlled microvalves to


Chapter based on the article:

Design guidelines and kinetic performance limits for spatial comprehensive three-dimensional chromatography for the analysis of intact proteins

Authors:

T. Themelis, J. De Vos, S. Eeltink

Analytical Chemistry 2022, 94, 13737–13744
6.1. Introduction

Since the conceptualization of spatial 3D-LC technology, there have been presented two different approaches that can be followed in order to establishing spatial 3D-LC separations. The first is to fully operate in the spatial domain, i.e., $^3\text{LC} \times ^3\text{LC} \times ^3\text{LC}$, where separated analytes are characterized by spatial ($X$, $Y$, $Z$) coordinates. In this mode of operation, confocal imaging techniques need to be applied to establish detection, limiting its applicability. Alternatively, the last development stage can be executed in the time domain ($^t\text{LC}$). In a $^4\text{LC} \times ^4\text{LC} \times ^4\text{LC}$ configuration, the analytes are eluted from the separation body prior to detection, enabling different detection methods [1]. The most straightforward of those involves immobilization of the effluent on a suitable substrate at regular intervals followed by mass-spectrometry imaging (MSI), making spatial 3D-LC amendable to proteomics profiling.

Theoretical studies using Pareto optimality have been carried out to assess the performance of different types of three-dimensional chromatographic systems, considering the maximization of peak capacity and minimization of the analysis times as the objectives [2,3]. In this study, small molecule ($< 1,500$ Da) separations in both time and spatial domains were studied using isocratic and gradient-elution modes. When considering interaction chromatography of biomolecules, such as peptides and proteins, there is a strong dependency between retention factor and mobile-phase composition. Consequently, in isocratic mode it can be anticipated that only a single analyte may elute at a specific solvent composition within a reasonable time frame, while others are either infinitely retained (or elute as a very broad peak at a much later time period) or will elute directly out of the separation body in the void time. This makes such an elution mode unsuitable for an $^4\text{LC}$ analysis. Gradient analysis of proteins and peptides is also incompatible with $^4\text{LC}$ separations, without the application of advanced refocusing strategies to maintain analytes within the separation channel as described by Themelis et al. [1], as one analyte may elute and reach already the outlet of the separation body, before the next analyte will elute. Another important aspect is that orthogonal separation mechanisms should be implemented in order to effectively utilize the available separation space and maximize peak capacity.

In the present paper, we discuss the pros and cons of integrating different separation modes in a spatial 3D-LC device targeting the analysis of intact proteins. Here, we consider design aspects of spatial 3D-LC devices incorporating an interconnected microchannels structure. This includes channel length and number of channels for each development. We also
revisit the kinetic performance limits for spatial 3D-LC, integrating ‘IEF × SEC × RPLC’ chromatographic modes using Pareto optimality.

6.2. Theory

According to the theory proposed by Giddings the maximum peak capacity of a multi-dimensional system can be calculated as the product of the individual peak capacities of each dimension, provided that orthogonal separation mechanisms are being used and that there are no losses due to undersampling [4]. Hence, the total peak capacity can be calculated from Eq. 2.26 which for a three-dimensional separation system becomes:

\[ n_{c}^{3D} = n_{c}^{1} \times n_{c}^{2} \times n_{c}^{3} \]  

(6.1)

Since all ‘fractions’ obtained after the 1D- and 2D-separation are developed in parallel in the 2D and 3D analysis respectively, the total separation time \( t^{3D} \) will be the sum of the individual separation times \( t \) of each development:

\[ t^{3D} = t^{1} + t^{2} + t^{3} \]  

(6.2)

For the analysis of intact proteins, the combination of isoelectric focusing, size-exclusion chromatography, and reversed-phase LC, leads to a powerful spatial 3D-LC solution, considering orthogonality and compatibility, see discussion in ‘Section 6.3.1.’. Below, the equations for the calculation of peak capacity and analysis times are presented for these different modes.

6.2.1. 1D-3LC: isoelectric focusing

The peak capacity in IEF with immobilized gradients was defined by Giddings as [5]:

\[ n_{c} = \left[ \frac{-F \cdot E \cdot (dq/dpH) \cdot L \cdot \Delta pH}{16RT} \right]^{1/2} \]  

(6.3)

where \( F \) is the Faraday constant (96485.3 C/mol·e\(^{-}\)), \( E \) the electric field strength, \( dq/dpH \) the rate of change of the electrophoretic charge in the pH gradient, \( L \) the channel length, \( \Delta pH \) the pH difference applied, \( R \) the ideal gas constant (8.314 J/K·mol), and \( T \) the temperature. The time to complete the separation process has been described by Zilberstain et al. as [6]:

\[ t = \frac{L \cdot k_{B} \cdot T}{q_{m} \cdot D_{m} \cdot E} \]  

(6.4)
with \( k_B \) being the Boltzmann constant \((8.617 \times 10^{-5} \text{ J/K})\), \( D_m \) the diffusion coefficient, and \( q_m \) the maximal value of net charge of the proteins. Eqs. (6.3) and (6.4) demonstrate a square root dependency of peak capacity and a linear dependency of analysis time with the length of the separation channel.

### 6.2.2. 3D-1 LC: size-exclusion chromatography

Fig. 6.1 shows a schematic overview of the SEC separation performed in the spatial domain (‘SEC). Under this condition, the development should be halted when the analyte that does not permeate the pores of the packing material (higher MW) reaches the end of the separation space, characterized by the length \( (L) \). An analyte fully permeating the pores will only reach a distance \( x_{f.p.} \) in the channel (of length \( L \)). Consequently, the effective separation space in ‘SEC corresponds to the distance \( L - x_{f.p.} \). The ratio of the distance that any retained analyte will cover over the total development length \( (x/L) \) conceptually coincides with the retardation factor \((R_F)\) in thin layer chromatography [7].

Peak capacity \((n_c)\) in ‘LC mode is defined as the number of peaks, characterized by a peak width \( W = 4\sigma \), that can fit with unity resolution in the available separation space. Consequently, in ‘SEC mode, \( n_c \) is described by the following equation:

\[
 n_c = 1 + \int_{x_{f.p.}}^{L} \frac{1}{4\sigma_x} dx 
\]  
(6.5)

The dispersion of a band in volumetric units is taken from Scott [8]:

\[
\sigma_v = \frac{V_0(1 + k_s)}{\sqrt{N}} 
\]  
(6.6)

where \( V_0(1+k_s) \) is equal to the retention volume \((V_R)\) and \( N \) the number of theoretical plates that according to Giddings can be considered to be constant along the chromatogram hence allows the use of Eq. 6.5 [9]. \( k_s \) is the retention factor relative to the molecule excluded from the pores which can be considered as a translation of the retention factor in adsorption chromatography, as reported by Neue and Ghrist et al. [10,11] and can be calculated from:

\[
k_s = m \cdot \log\left(\frac{\text{MW}_e}{\text{MW}}\right) 
\]  
(6.7)

with \( \text{MW} \) being the molecular weight of the analyte of interest, \( \text{MW}_e \) the molecular weight at the point of exclusion, and \( m \) a constant that is specific for the packing material.
Equation 6.7 can be derived from the equation describing the linear part of a calibration curve in SEC which can be described as:

\[ K_D Y + \beta = \log(MW) \]  \hspace{1cm} (6.8)

where \( \beta \) is the intercept corresponding to the molecular weight of exclusion \((MW_e)\) and \(Y\) is the slope [12]. \( K_D \) is the thermodynamic retention factor, which is the fraction of the intraparticle volume that is accessible to the analyte. It also has been reported that the retention factor \( k_s \) can be described as:

\[ k_s = K_D \cdot \frac{V_i}{V_0} \]  \hspace{1cm} (6.9)

with \( V_0 \) being the interstitial volume and \( V_i \) the intraparticle volume [12]. By rearranging (6.8) we obtain the following:

\[ K_D Y = \log(MW) - \log(MW_e) \]  \hspace{1cm} (6.10)

and by solving (6.9) for \( K_D \), substituting it in (6.10) and rearranging, we obtain:

\[ k_s = -\frac{V_i}{V_0} \cdot \frac{1}{Y} \cdot \log\left(\frac{MW}{MW_e}\right) \]  \hspace{1cm} (6.11)

By defining a constant \( m = -(V_i/V_0)(1/Y) \), we reach the final form defined as Eq. 6.7.

To insert Eq. 6.6 in 6.5, Eq. 6.6 needs to be transformed in spatial units via the relationship \( \sigma_x = \frac{\sigma_v x}{v_R} \), and Eq. 6.5 becomes:

\[ n_c = 1 + \int_{x_{f.p.}}^{L} \frac{\sqrt{N}}{4x} dx \]  \hspace{1cm} (6.12)

By changing the integration variable to \( R_F \) and the integration limits accordingly, the above equation transforms into:

\[ n_c = 1 + \int_{R_{F,f.p.}}^{1} \frac{\sqrt{N}}{4R_F} dR_F \]  \hspace{1cm} (6.13)

where \( R_{F,f.p.} \) is the retardation factor of the analyte fully permeating the pores of the stationary phase. From the above equation after integration, we obtain:

\[ n_c = 1 + \frac{\sqrt{N}}{4} \ln \left( \frac{1}{R_{F,f.p.}} \right) \]  \hspace{1cm} (6.14)

Furthermore, it is known that the \( R_F \) and \( k_s \) are connected by the following relationship:

\[ R_F = \frac{1}{1 + k_s} \]  \hspace{1cm} (6.15)
and by inserting Eqs. (6.7) and (6.15) in (6.14) we reach the final form for the calculation of peak capacity in xSEC:

\[ n_c = 1 + \frac{\sqrt{N}}{4} \ln \left[ 1 + m \cdot \log \left( \frac{\text{MW}_{f.p.}}{\text{MW}_{e}} \right) \right] \quad (6.16) \]

where MW\text{f.p.} is the molecular weight of a fully permeating analyte. The value of N in the above equation can be obtained from a variation of the plate-height model proposed by Engelhardt et al.\cite{13} starting from:

\[ H = a \cdot d_p + b \cdot \frac{(1+k_s)D_m}{u_z} + c \cdot \frac{k_s^2}{(1+k_s)^2} \cdot \frac{d_p^2}{D_m} u_z \quad (6.17) \]

where \( u_z \) is the interstitial velocity and A, B, C are expressed via their respective reduced terms such as \( A = \alpha \cdot d_p, B = b(1+k_s)D_m \) and \( C = c \cdot k_s \cdot d_p^2/(1+k_s)^2 \cdot D_m \), with B and C showing dependency on the retention factor. Equation 6.17 can be transformed in its reduced form:

\[ h = a + \frac{b(1+k_s)}{u_z} + c \cdot \frac{k_s^2}{(1+k_s)^2} u_z \quad (6.17b) \]

where \( h \) is the reduced plate height (\( h=H/d_p \)) and \( u_z \) the reduced interstitial velocity (\( u_z = u \cdot d_p/D_m \)) and \( a, b \) and \( c \) the reduced parameters related to eddy diffusion, molecular diffusion, and mass-transfer, respectively. By solving Eq. 6.15 for \( k_s \) and substituting it in (6.17b) we obtain Eq. 6.18 which used to calculate \( H \) and consequently \( N \) in xSEC.

\[ h = a + \frac{b}{u_z R_F} + c(1 - R_F)^2 u_z \quad (6.18) \]

The analysis time for such a separation that is halted when the unretained analyte reaches the channel outlet, can be calculated from the dead time of the column:

\[ t_0 = \frac{L}{u_z} \quad (6.19) \]

considering the interstitial velocity which can be related to the Darcy equation (2.1) used for the calculation of pressure drop (\( \Delta P \)) in a system as follows:

\[ \Delta P = \frac{u_z \epsilon_\epsilon \phi \eta L}{\epsilon_T d_p^2} \quad (6.20) \]

where \( \epsilon_T \) is the total porosity, \( \epsilon_\epsilon \) the external porosity, \( d_p \) the particle size, \( \phi \) the flow-resistance factor, and \( \eta \) the mobile-phase viscosity.
Figure 6.1. Schematic representation of the bands of the least and most retained analytes in a size-exclusion chromatography column operated in spatial mode. The development should be halted when the least retained analyte reaches the outlet, which restricts the effective separation space to a region equal to $L-x_f$.

6.2.3. 3D-LC: gradient reversed-phase liquid chromatography

To estimate the peak capacity of gradient RPLC for intact proteins utilized in the 3D development stage in the time domain, Eq. 6.21 as established by Neue and Gilar has been used [14]:

$$n_c = 1 + \frac{\sqrt{N}}{4} \cdot \frac{S \cdot \Delta c}{S \cdot \Delta c (t_0 / t_G) + 1}$$

(6.21)

where $N$ is the number of theoretical plates, $S$ solvent-strength parameter, $\Delta c$ the gradient span, and $t_G/t_0$ represents the gradient steepness defined as the ratio of the gradient time ($t_G$) and the column dead time ($t_0$). This equation represents a simplified form of Eq. 3.2 and is applicable to large (bio-)macromolecules. It results from the assumption that peak widths along the chromatogram are equal and from the fact that typical values of $b$ for proteins can be significantly higher due to their characteristic “on/off” retention mechanism which reflects of much higher $S$ values. $N$ is linked to the reduced plate height ($h$), with $h = H / d_p$, and varies as function of the reduced linear velocity with $v = u_0 \cdot d_p / D_m$, where $u_0$ refers to the velocity of an unretained compound that is able to fully penetrate the porous particles [15]. For the calculation of $h$, the van Deemter equation in its dimensionless form (Eq. 2.12) has been used, with $a$, $b$, $c$ representing the reduced eddy dispersion, longitudinal, and mass-transfer contribution coefficients respectively. The analysis time for the 3D development is the sum of the column dead time ($t_0$) and the gradient time ($t_G$) applied, considering the absence of gradient
delay time. $t_0$ is related to the linear velocity through Eq. 6.19 considering $u_0$ instead of $u_z$, which can be retrieved from the Darcy equation (Eq. 2.1).

### 6.2.4. Effect of microchannel structure on resulting peak capacity determination

In a spatial 3D-LC microdevice that contains a discrete number of 2D- and 3D-channels, sample transfer between dimensions impacts the total peak capacity that can be generated. Below, the explanation that is provided describes the impact of the sample transfer interface between the 1D and 2D development stage, see Fig. 6.2A, but a similar methodology is applicable for sample transfer between the 2D and 3D development stage. Two different cases can be distinguished. Case i) is when the peak width ($4\sigma$) after the 1D-development is larger than the distance between two consecutive 2D channels (Fig. 6.2B).

**Figure 6.2.** Top view of the 1D- to 2D-interface with staggered channel design in a spatial 3D-LC microdevice. Insert: B) shows the case when the band width ($4\sigma$) is larger than the distance between two consecutive 2D channels ($\alpha$) and C) shows the case when the band width ($4\sigma$) is smaller than the distance between two consecutive 2D channels and the peak is positioned exactly at the outlet the 2D flow distributor channel.

This process shares some basic similarities with the modulation encountered in conventional comprehensive MD-LC and a single peak is sampled at least two times, as the band will be injected in at least two 2D channels [16]. Vivó-Truyols et al. considered the second dimension separation as the detector of the 1D effluent and hence the sampling rate as an analogy of the detection frequency, in order to be able to calculate the effect of the sampling rate on the obtained peak capacity of the preceding dimension [15]. In a similar way of thinking we used the concept of whole column imaging detection (real time visualization of the whole channel...
using a number of optic fibers to illuminate the channel at evenly spaced intervals [16]) to perform the same calculation and thus the ¹D the peak capacity was derived to be:

\[ n_c = \frac{L}{4 \sqrt{(\frac{\sigma_{peak}}{\alpha})^2 + (\frac{\delta_{det}}{\alpha})^2}} \]  

(6.22)

where \(1/\alpha\) is the detection frequency with \(\alpha\) in spatial units being equal to the distance between the two consecutive “detection points” (²D channels), and \(\delta_{det}\) is a constant equal to 4.76 according to Davis et al. [17].

Case ii) is when the \(4\sigma\) of a band after the ¹D-analysis is smaller than the distance between two consecutive channels, see Fig. 6.2C. Two possible situations can occur: a) that the band is positioned such that it splits and is injected in two ²D-channels. The probability that this event happens can be calculated as \(4\sigma/\alpha\). Case b) is that the band is positioned such that it is directed towards only a single ²D-channel. This probability is equal to \(1-(4\sigma/\alpha)\). By taking these two individual probabilities, and extending this for every tract of the ¹D channel delimited between two consecutive ²D channels, we can calculate \(n_c\) as:

\[ n_c = \left[ \left(\frac{1}{2}\right) \cdot \frac{4^1\sigma_{peak}}{\alpha} + 1 \cdot \left(1 - \frac{4^1\sigma_{peak}}{\alpha}\right) \right] \cdot \frac{L}{\alpha} \]  

(6.23)

that can be simplified to its final form:

\[ n_c = \left(1 - \frac{2^1\sigma_{peak}}{\alpha}\right) \cdot \frac{L}{\alpha} \]  

(6.24)

6.3. Experimental section

To assess the performance of a spatial 3D-LC chromatographic system, incorporating ³IEF × ³SEC × ³RPLC and targeting the analysis of intact proteins, a Pareto optimality study was executed. The script was developed in-house and was written in MATLAB (R2019b, The Mathworks, Natick, USA). In a first step, the above defined Eqs. are used to calculate all possible \(n_c\) and \(t\) values considering the ranges of parameters in Table 6.I for ³IEF, ³SEC, and ³RPLC, respectively. In the next step, the total peak capacity for the spatial 3D-LC mode is determined using all possible combinations created in the previous step, and then the Pareto optimal front is established for spatial 3D-LC. For a more detailed view of the methodology, the reader is directed to the literature by Massart and Vandeginste and Vivó-Truyols et al.[15,18].
The microdevice was constructed from cyclic olefin copolymer (COC, TOPAS advanced polymers, grade 8007, Kunststoff-Zentrum, Leipzig, Germany) using a computer-numerical-control micro-milling machine (Datron M7 Compact, Mühltal-Traisa, Germany). Prior to milling the microchannels, a face milling step was applied to reduce the surface roughness. The 2D-flow distributors and microchannels as well as the 3D-flow distributor channels were constructed by using a 200 μm diameter single-flute endmill and the 1D-channel using a 250 μm diameter ball-nose milling tool. A 200 μm diameter drill was used to drill the connection holes between the chip layers as well as the 3D-channels. An irreversible bond between the various substrates was obtained via solvent-vapor-assisted bonding. After

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Parameter, Symbol (Eq.)</th>
<th>Value (units)</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>1D-IEF</td>
<td>Electric field strength, $E$ (6.3,6.4)</td>
<td>$5 \text{ (V}\cdot\text{m}^{-1})$</td>
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<tr>
<td></td>
<td>Channel length, $L$ (6.3,6.4)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pH range, $\Delta p$H (6.3)</td>
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</tr>
<tr>
<td></td>
<td>Rate of change of electrophoretic charge in the pH gradient, $dq/dp$H (6.3)</td>
<td>$-9 \text{ (/)}$</td>
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<td></td>
<td>Diffusion coefficient of a protein of 900 kDa, $D_m$ (6.4)</td>
<td>$2.6\cdot10^{-11} \text{ (m}^2\cdot\text{s}^{-1})$</td>
<td>[11]</td>
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<td></td>
<td>Maximal value of net charge of the proteins, $q_m$ (6.4)</td>
<td>$20 \text{ (/)}$</td>
<td>[6]</td>
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<tr>
<td>2D-SEC</td>
<td>van Deemter coefficient, $a$ (6.18)</td>
<td>$2 \text{ (/)}$</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>van Deemter coefficient, $b$ (6.18)</td>
<td>$2 \text{ (/)}$</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>van Deemter coefficient, $c$ (6.18)</td>
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<td>[19]</td>
</tr>
<tr>
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<td>Column external porosity, $e_e$ (6.20)</td>
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<td>[8]</td>
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<tr>
<td></td>
<td>Column total porosity, $e_T$ (6.20)</td>
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<td>[8]</td>
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<td>Flow-resistance factor, $\phi$ (6.20)</td>
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<td>Particle diameter, $d_p$ (6.20)</td>
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<td></td>
<td>MW range</td>
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<td>Mobile-phase viscosity, $\eta$ (6.20,2.1)</td>
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<td>$M_W f_p$ of the analyte fully permeating the pore (6.16)</td>
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<td>Max. pressure drop, $\Delta P$ (6.20)</td>
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<td>3D-RPLC</td>
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<td>Channel length, $L$ (2.1)</td>
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<td>$t_{cd}/t_0$ (6.21)</td>
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<td></td>
<td>$S-Ac$ (6.21)</td>
<td>$20 \text{ (/)}$</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>Max. pressure drop, $\Delta P$ (2.1)</td>
<td>$2\cdot10^7 \text{ (Pa)}$</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.I. Parameters and range of variables used to compute peak capacities and analysis times using Pareto optimization.
Chapter 6

exposing the top substrate to cyclohexane vapor, bottom and top substrates were aligned with a precision of approximately 2 μm using an in-house, custom-made, aluminum holder, and pressed together using a hydraulic press for 45 min.

6.4. Results and discussion

6.4.1. Selection of separation modes

Numerous separation modes and their combinations were considered as possible candidates to be integrated in the spatial 3D-LC device. Isocratic and gradient RPLC, ion-exchange chromatography (IEX), SEC, hydrophobic interaction chromatography (HIC), and hydrophilic interaction liquid chromatography (HILIC) were examined amongst the pressure-driven methods, while IEF, capillary electrophoresis (CE), and capillary gel electrophoresis (CGE) were considered as electro-driven methods. As analytes should be retained within the separation body during the 1D- and 2D (single column-volume development), LC modes that are operated under gradient-elution conditions, such as RPLC, IEX, HIC, and HILIC, are difficult to be integrated in spatial separations unless elaborate types of gradients are applied (single column-volume gradient) or specific stationary phases with incorporated hydrophobicity gradient are synthesized. Since the 3D is performed in the time domain, the aforementioned modes were considered only for the 3rd dimension. The rather high salt concentration though used in IEX and HIC increases significantly the risk of clogging the microchannels in a spatial 3D-LC device, making these two modes slightly less attractive candidates compared to RPLC and HILIC modes. Isocratic modes will be counterproductive in spatial separations. This is due to the steep dependency between retention factor and mobile phase composition observed in protein analysis that under isocratic conditions would lead to the majority of analytes being stuck at the column head and just a few of them immediately eluting towards the column outlet (on/off retention mechanism). SEC, despite of the fact that is an isocratic method, has a very well defined limit to its separation capacity, due to the fact that the peaks are confined to a definite separation volume. Hence, this approach would eventually spread the analytes based on their MW along a portion of the channel and consequently constitutes a good choice for the 1D and 2D but not an ideal one for the 3D due to its rather low efficiency. When it comes to electro-driven modes, IEF represents an ideal solution for space-based separations as it can provide high resolution in a single column-volume analysis. This reason together with the fact that potential mobilization of the analytes
towards a detector, after the end of the focusing process, would lead to loss of resolving power
drove us to consider this mode only as an option for the 1D and 2D. With CGE in the 1D and 2D
(performing in non-denaturing conditions given that the use of SDS would potentially impact
the following dimensions especially when hydrophobic stationary phases are used) high
resolution can be potentially achieved. Nonetheless, it was not chosen amongst the considered
separation modes because it carries a major drawback which is the fact that the gel would get
fractured in the cases where a pressure-driven mode follows and. Finally, CE constitutes an
attractive option for all three dimensions, even though a loss of the available separation space
must be considered in the 1st and 2nd dimension to go with considerable technical difficulties
of incorporating an array of electrodes in the 3D channels outlets. An overview of the strengths
and weaknesses of the most feasible combinations of separation modes that were considered,
targeting protein analysis in a spatial 1L C× 1L C× 1L C configuration is provided in Table 6.II.
The upper half of each cell shows the evaluation of the 1D/2D combination and the lower half
the 2D/3D combination. The main factors that have been considered include orthogonality and
mobile-phase compatibility for subsequent developments (solubility, precipitation, and sample
break-through). Furthermore, the possibility to preconcentrate and compress peaks prior to the
next development via the application of stationary-phase focusing and peak compression
induced by the use of a stronger solvent, [22] as well as solvent compatibility with respect to
MS detection has been taken into account. The abovementioned considerations were graded
(maximum total score = 20) considering the 1D/2D interface and the 2D/3D interface,
respectively. For orthogonality a maximum score of 3 could be attributed as this greatly
influences the maximum peak capacity that can be generated. For mobile-phase compatibility
a weight factor of 4 was assigned because solvent compatibility is a key requirement and
incompatible mobile phases always lead to failure in separation. For stationary-phase focusing
and MS compatibility (considering spotting and MS imaging) values of 1 and 2 were accounted
respectively. Regarding MS compatibility, it has been reported that in analysis of biological
systems, inorganic salts are also part of protein extracts and can be removed by washing the
dried-droplet MALDI spots with cold water [23]. Consequently, even for seemingly MS
incompatible mobile-phase systems, such as HIC or IEX, detection is still feasible. To
pronounce even more incompatible cases where one of the sub-scores equals 0, the total score
was penalized and reduced with a value corresponding to the maximum weight factor that could
have been earned.

Table 6.II. Evaluation of various combinations of separation modes in a spatial 1L C× 1L C× 1L C configuration.
### Chapter 6

- **SEC × IEF**
- **SEC × CE**
- **IEF × SEC**
- **IEF × CE**
- **CE × SEC**
- **CE × IEF**

<table>
<thead>
<tr>
<th></th>
<th>Orthogonality</th>
<th>MP compatibility</th>
<th>SP focusing</th>
<th>MS compatibility</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RP</strong></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>18/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 2/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>17/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>17/20</td>
</tr>
<tr>
<td><strong>IEX</strong></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 3/4</td>
<td>SP focusing: 1/1</td>
<td>MS compatibility: 1/2</td>
<td>14/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 1/2</td>
<td>12/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 1/2</td>
<td>12/20</td>
</tr>
<tr>
<td><strong>HIC</strong></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 3/4</td>
<td>SP focusing: 1/1</td>
<td>MS compatibility: 1/2</td>
<td>16/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 1/2</td>
<td>15/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 1/2</td>
<td>15/20</td>
</tr>
<tr>
<td><strong>HILIC</strong></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 3/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>8/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>8/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 3/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>8/20</td>
</tr>
<tr>
<td><strong>CE</strong></td>
<td>Orthogonality: 2/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>15/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 2/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>15/20</td>
</tr>
<tr>
<td></td>
<td>Orthogonality: 2/3</td>
<td>MP compatibility: 4/4</td>
<td>SP focusing: 0/1</td>
<td>MS compatibility: 2/2</td>
<td>15/20</td>
</tr>
</tbody>
</table>
To better understand the scoring process, we take as an example the combination of SEC×IEF×IEX. This was split in two parts, namely SEC×IEF for the 1D/2D interface and IEF×IEX for the 2D/3D interface. The first combination of mechanisms (SEC×IEF) has completely different separation principles hence it provides very high orthogonality and for this reason the attributed score was 3/3. Both these two separations modes are performed under aqueous conditions and demonstrate very good mobile phase compatibility thus a grade of 4/4 was assigned in this attribute. When passing from the 1D-SEC to 2D-IEF there is no solvent induced focusing at the head of the 2D channels so the stationary phase focusing scored 0/1 and because of that, 1 point will be subtracted from the total score of the overall combination at the end. When it comes to MS detection, no compatibility issues should be present for this specific combination of separation modes, hence it was graded with a 2/2. Passing to the 2D/3D interface, the orthogonality between IEF and IEX is low due to the fact that they are both charge based separations, so an 1/3 score was given. Nevertheless, their mobile phase compatibility will be rather good due to the aqueous nature of both mobile phases (score 3/4). Stationary phase focusing was graded 1/1 because proteins at their pI will be retained on an IEX stationary phase and finally, for the present combination the use of a salt gradient in the 3D-IEX will reduce its MS compatibility and this is the reason why the score was 1/2. Consequently, a total score of 14/20 was given to SEC×IEF×IEX.

As a result, we have selected for the Pareto optimization the combination of IEF×SEC×RP, being the one that obtained the highest score together with SEC×IEF×RP. We opted for IEF for the 1D development, as it provides higher loadability compared to SEC. SEC instead was chosen for the 2D (‘LC) development because of the high orthogonality and good mobile phase compatibility with IEF, and RPLC for the 3D (‘LC) development given the excellent resolving power, high orthogonality and good mobile phase compatibility with the prior dimension.

6.4.2. Considerations on channel length and particle diameter

The optimal length of the 1D-, 2D-, and 3D-separation segments / channels depends on the needs of the chromatographer aiming at either maximizing peak capacity or sample throughput. Fig. 6.3A displays Pareto fronts of the individual 1IEF, 1SEC, and 1RPLC developments and how the respective peak capacities vary as a function of channel length. To maximize $n_c$ at a specific maximum pressure ($\Delta P = 200$ bar), the Pareto optimal front of 1SEC
and 1RPLC only accounts for the smallest particle-diameter values possible, which was 1.5 μm (see Fig. 6.3A dashed lines corresponding to the dashed secondary y-axis). This can be readily justified when considering the low diffusion coefficients of large biomolecules, which permit the operation of the spatial device at rather low ranges of linear velocities, without compromising the separation with band broadening induced by longitudinal diffusion. Performing the separation at low linear velocities means that there is an amount of available backpressure for the system that is not being used and which can be used to increase the efficiency by reducing the applied particle size at the minimum available value for all the different channel lengths that are considered in this study. The maximum peak capacity that can be generated in 1SEC mode is relatively modest, and this is mainly the result of the limited separation space inside the microchannel, corresponding to a distance $L - x$ (Fig. 6.1). Increasing the channel length from 1 to 5 cm leads to a 131% increase in peak capacity (from 6 to 14) as can be seen from Fig. 6.3 represented by blue line and triangles. Increasing the separation space from 1 to 5 cm in 3IEF leads to an increase in peak capacity from 27 to 62 (127%), (see red line and diamonds). Fig. 6.3 (orange line and squares) demonstrates that the largest gain in $n_c$ (increase from 17 to 82) can be realized when increasing the separation space (channel length) in 1RPLC, as $n_c$ almost linearly increases with the length in the range applied. Nevertheless, when considering reduced van Deemter coefficients accounting for the difficulties encountered in packing microchannels ($a = 2$, $b = 1.18$, $c = 0.37$ from ref. [24] for packing with 3 μm diameter, C18 particles) the total peak capacity in RPLC will be reduced by approximately 30%, (see black line and crosses) and the respective gain will be 363%.

Figure 6.3. Pareto fronts of length vs. peak capacity (full lines) and particle size vs. peak capacity (dashed lines) for IEF in 1D (red, diamonds), SEC in spatial mode in 2D (blue, triangles), and gradient RPLC elution in 3D (orange, squares) for $a$, $b$, $c$ values corresponding to a commercially packed column, shown in Table 6.I and gradient RPLC elution in 3D (black, crosses) for $a$, $b$, $c$ values corresponding to a packed chip, adopted from ref [24].
6.4.3. Effect of the number of 2D and 3D channels on sample transfer and resulting peak capacity

In a spatial 3D-LC device that incorporates a discrete number of channels in the 2D and 3D, the number and the distance between the channels (α) in correlation with the peak width obtained during the preceding separation, will affect the total peak capacity. Let us consider the interface between IEF and SEC and make the logical assumption that diffusional broadening during the transfer of analytes to the next dimension is not an issue due to the focusing induced in 1D (and of little concern in SEC/RP interface because of the lower diffusion coefficient of the molecules). The channel length of IEF is systematically varied in this Pareto optimality study, yielding a range of nc values which correspond to different peak widths (4σ). Next, α (which is related to the number of 2D-channels) is systematically varied in the range of 0.05-15 mm and compared to 4σ. When the peak widths are narrower compared to α (situation Fig. 6.2C), “undersampling” will occur and the corrected peak capacity (<nc>, incorporating the loss due to undersampling) is calculated by Eq. 6.24. This implies that the peak capacity gained during the preceding separation will be partially lost as separated peaks will be remixed at the start of subsequent development (Fig. 6.4, dashed blue line). Consequently, the ratio of the corrected peak capacity over the maximum peak capacity (<nc>/nc) remains below 0.5 corresponding to a loss in peak capacity of minimum 50%. When α is reduced, the number of parallel 2D channels is increased (4σ becomes larger than α), hence <nc> will now be calculated via Eq. 6.22. As a result, it can be seen in Fig. 6.4 (full line, full symbols) that <nc>/nc increases approaching 1 towards the situation that there is no loss in peak capacity. It is important at this point to clarify that the lines represent only the Pareto-fronts of the minimum number of channels required in order to achieve each <nc>/nc value and not the total number of solutions obtained from our calculations of the different combinations of channel lengths and distances in the 1D/2D interface. Now let us consider the interface between SEC and RPLC. Generally, the peak width in SEC is larger than in IEF, thus a relatively higher α and a lower number of 2D-channels is sufficient in order to maintain the obtained maximum peak capacity, see Fig. 6.4. For a <nc>/nc of 0.9, ideally 250 2D-channels (′nc = 62 for a 5 cm channel length) are required while almost 100 3D channels - per - 2D-channel (′2nc = 13 for a 5 cm channel) are needed, leading to a device containing 25,000 3D-channels. This implies that the 2D-channel diameter equals 100 µm (with inter-channel distance equal to 100 µm). For the 3D-channels, a diameter up to 250 µm would be applicable, however, smaller
channel diameters may be preferable to overcome chromatographic dilution, and to establish the envisioned interfacing with MSI.

**Figure 6.4.** Curves displaying the ratio of the corrected total peak capacity \( \langle n_c \rangle \) (considering losses in peak capacity due to sample transfer) and the theoretical total peak capacity \( \langle n_c \rangle / n_c \) vs. the number of channels in the following dimension. The blue lines and square symbols represent the Pareto front for the interface between 1D and 2D while the orange lines and square symbols the Pareto front for the interface between 2D and 3D. The solid line and full symbols describe the case when \( \alpha \leq 4\sigma \) and \( n_c \) is calculated via Eq. 6.22 while the dashed lines and open symbols stand for the case when \( \alpha > 4\sigma \) and \( n_c \) is calculated via Eq. 6.24.

### 6.4.4. Effect of prototyping constraints on resolving power

Fig. 6.5 shows Pareto fronts, visualizing the kinetic performance limits in terms of analysis time and resulting peak capacity, that can be established for \(^4\)IEF \( \times \) \(^4\)SEC \( \times \) \(^4\)RPLC devices integrating an interconnected microchannel structure. The red full curve is the optimality front of all the combinations of the parameters reported in Table 6.1, when considering inter-channel distances (\( \alpha \)) in the range of 0.4-1.5 mm (and hence the respective channel i.d.) that can be achieved with the current state-of-the-art micromilling and solvent-vapor-assisted-bonding technology, as utilized for the fabrication of spatial 2D-LC and 3D-LC microchips in previous studies performed by our group \([3,25,26]\). The red dashed line in Fig. 6.5 shows the Pareto front, considering the effect of packing quality on resulting performance (as reported for microchips in \([23]\)). For all devices, the peak capacity increases almost linearly with analysis time. Fig. 6.6 shows the channel layout of all the modules combined to build a microfluidic chip device for comprehensive spatial 3D-LC containing sixty-four 200 µm i.d.
2D-channels and a total of four-thousand-ninety-six 200 µm i.d. 3D-channels, that should generate a peak capacity of 32,600 within 44 min of total analysis time (the position on the curve is marked by a black asterisk in Fig. 6.5).

When considering a lower packing quality, the total peak capacity amounts to 23,200 in 44 min (position marked by double asterisk). When further increasing the number of 2D-channels to 125 by decreasing channel diameters to 100 µm spaced 300 µm from each other, a maximum peak capacity of 40,000 would be feasible within 53 min of analysis time. Using high-end chip prototyping facilities (two photon polymerization 3D printing, electron-beam lithography etc.) that allow to reduce the inter-channel distances (in the range of 0.05-0.4 mm) and create 25 µm i.d. channels spaced 25 µm from each other, the peak capacity can be significantly improved, see the black solid and dashed lines in Fig. 6.5. When 1IEF, 3SEC, and 3RPLC are successfully implemented in the 1D-, 2D-, and 3D-development stages, this could theoretically lead to a device yielding 54,300 in peak capacity with a total analysis time of 53 min for a device with ideally packed 3D channels and 35,200 for a device accounting for lower efficiency due to issues related with microchannel packing. To put the aforementioned peak capacity values in perspective, the resolving power of such a device would be comparable to what 2D gel electrophoresis can achieve, a technique that has shown to be able to separate 10,000 proteins.
in a gel. Nevertheless, in 2DGE these values are obtained after several hours of analysis times while spatial 3D-LC could achieve this type of separation with much higher throughput within one hour of separation time using the discussed chip designs. When increasing the footprint of the device higher peak capacity values and of course higher analysis time can be expected but even in such case it would be comparable or even lower than 2D gel electrophoresis.

![Microfluidic chip for spatial 3D-LC](image)

**Figure 6.6.** Microfluidic chip for spatial 3D-LC that can generate a peak capacity of 32,600 within 44 minutes of analysis time. A) “Exploded” view of the different modules combined to create a spatial 3D-LC device. From top to bottom there are: the 3D flow distributor (close-up in B), the two symmetrical plates containing the 2D flow distributor and flow collector, one 1D channel, 64 2D channels (close-up in C), and the bottom module containing 4096 3D channels (close-up in D). E) Photograph of a prototype chip device for spatial 3D-LC after solvent assisted bonding.

### 6.5. Concluding remarks

In the present work, several factors have been studied that must be accounted for the design of a spatial 3D-LC micro-device with specific characteristics regarding a realistic selection of orthogonal separation mechanisms. First, different combinations of separation modes have been evaluated and IEF × SEC × RPLC has been chosen as the most fitting one for the purpose of protein analysis. Proper equations for the calculation of peak capacity values were used, considering the particular nature of spatial 3D-LC technology and specifically the 1D- and 2D-analysis which are performed in the spatial domain. Via these equations the effect of the selection of channel length on separation efficiency was studied. Additionally, the influence that the transfer of analytes from one dimension to the following has on the total efficiency of the chip was assessed. Based on that, it was determined that a device with one 1D-, 250 2D- (channel length 5 cm, i.d. 100 µm) and 25,000 3D-channels (channel length 5 cm,
i.d. 250 μm) would suffer almost no loss in peak capacity due to inefficient sample transfer. Two different chip designs, including a device that is possible to build with the currently available technology in micromachining, were compared in terms of efficiency. A prototype device was constructed using in-house microfabrication techniques, that could potentially achieve a maximum total peak capacity of 32,600 in only 44 minutes after the implementation of the proposed separation modes. By using state-of-the-art chip manufacturing technology, this device could be improved to showcase an interconnected channel design that can theoretically achieve a peak capacity of 54,300 in 53 minutes of total analysis time.

6.6. References


7.1. Conclusions

The need for separation methods that can offer high resolving power and throughput has always been the main driving force of development for the field of separation science. The present doctoral thesis reports a series of studies aiming to increase chromatographic efficiency through the development of fully optimized unidimensional chromatographic methods as well as of technologies aiding the realization of a novel multidimensional separation concept, namely spatial three-dimensional chromatography.

First, kinetic plot modelling was used to assess the performance limits of a 1D-LC approach and optimize the separation of complex antibiotics samples through the use of state of the art fused core shell particle columns operated at 1500 bar. Scouting runs utilizing a 100 mm column allowed for the generation of free length kinetic plots which predicted the expected performance and hence the required column length to achieve the needed peak capacities for full elucidation of the complex sample as well as the analysis times expected when elongating columns to reach the kinetic limit. These predictions concurred nicely with experimental data where we observed values of peak capacity equal to 210 for a 200 mm coupled column operated at a $t_G/t_0 = 20$ and 400 for a 400 mm coupled column operated at $t_G/t_0 = 40$ which could fully resolve 54% and 73% of the total compounds present in the sample respectively. It was observed that, even at the KPL conditions, gradient occupation was reduced with increasing $t_G$ and flow rate. Thus, a detailed theoretical explanation was provided on how the modulation of the parameters that influence gradient steepness ($\Delta c$, $t_0$, $t_G$) affects gradient occupation. Subsequently, we provided a tool in which the optimal $\Delta c$ can be determined and adjusted together with the gradient time in order to maintain a fixed $\Delta c / t_G$ and achieve a gradient occupation of 1 leading to shorter instrument cycles and maximized throughput.

With the need for higher separation efficiencies being evident, spatial 3D-LC was introduced having unarguably very high potential to offer in the field of separation sciences in life science research. A tutorial containing fundamental ideas to assist the realization of a microfluidic device for spatial MD-LC has been presented. After having already successfully demonstrated the initial step of prototyping via micromilling and solvent assisted bonding, the major challenges that must be tackled before obtaining a fully functional spatial 3D-LC device were discussed. One of these bottlenecks is the issue of flow confinement i.e., the ability to maintain the flow and the analytes inside the predetermined separation space devoted to each individual dimension and hence avoid leakages between dimensions that would compromise
the quality of the separation. Different proposed solutions mainly for the \(1D/2D\) interface but also some theoretical approaches for the \(2D/3D\) interfaces have been presented. Another important aspect that was discussed is the implementation of the device with different orthogonal separation mechanisms both of pressure and electro-driven nature in the three dimensions. In this context, \textit{in-situ} synthesis of organic monoliths has been discussed as the most straightforward way of integrating stationary phases, considering the challenges associated with the packing of thermoplastic devices having multiple interconnected channels. The last step towards a fully operating spatial 3D-LC device is coupling it to a detection system.

Considering the crucial role that MS plays in modern life sciences due to the additional resolving power that can provide as well as because of its high sensitivity, it is regarded as the most suited approach for establishing detection. MALDI MS imaging has been proposed as the best technique when aiming to perform MS detection but also maintaining the spatial resolution of the analytes separated in the microdevice. This is envisioned to be achieved by depositing the effluent of the third dimension on a MALDI plate mounted on a X-Y-Z motorized stage programmed to move at the appropriate time and space intervals.

In the following part of the thesis some of the discussed ideas on flow control and confinement were tested. Two different designs of bifurcated flow distributors for spatial MD separations were experimentally studied, one with 175\(^\circ\) angle between the bifurcations and a second one featuring an angle of 180\(^\circ\) (both designs were previously introduced and studied via CFD). It has been concluded that an angle of 180\(^\circ\) leads to a nearly perfect homogenous flow profile across all parallel channels, independent of the flow rate applied (50-1000 \(\mu L/min\)). Furthermore, two different approaches to confine the flow of the \(1D\) in the appropriate separation space were explored. The integration of physical barriers including cross-section reduction and localization of monoliths in the \(2D\) was considered as an option to avoid leakages. This was successfully applied in the \(1D/2D\) interface (could eventually be used in the \(2D/3D\) interface) but minor leakages were observed even at lower back pressures. For the same purpose, an active-valving approach was also successfully developed and used in combination with stationary-phase focusing between the developments. This mechanism allows to completely avoid lateral leaks for backpressures up to 50 bar and hence to fully utilize the separation space provided by the chip, maximizing the potential peak capacity. The low recovery of the sample when injected from the \(1D\) to the \(2D\), can be addressed by increasing the number of \(2D\) channels. Achieving focusing/remobilization between the different dimensions is crucial for the operation of a spatial device since it not only reduces dilution of the peaks but
also permits to tune the dimension of the channels accordingly to the needs for a possible hyphenation with MS imaging detection.

Finally, for the last part of the thesis several design aspects that must be taken into consideration for the realization of spatial 3D-LC device have been studied. Setting as a goal the analysis of intact proteins, different combinations of separation mechanisms have been evaluated and $^3\text{IEF} \times ^3\text{SEC} \times ^3\text{RPLC}$ has been chosen as the most fitting one. To be able to correctly calculate the performance limits of such a device we derived an equation that accounts for the SEC operated in spatial mode in the $^2\text{D}$ and the relatively lower values of peak capacity that this can yield compared to time based SEC. Using this equation (Eq. 6.16), together with the respective ones for $^1\text{IEF}$ (Eq. 6.3) and $^1\text{RPLC}$ (Eq. 6.21) and by applying a Pareto optimality approach we investigated the effect of channel length on separation efficiency. Another key aspect that influences the total efficiency of the device is the transfer of analytes from one dimension to the following. A thorough investigation on this aspect was carried out and we concluded that a device with one $^1\text{D}$-, 250 $^2\text{D}$- (channel length 5 cm, i.d. 100 $\mu$m) and 25,000 $^3\text{D}$-channels (channel length 5 cm, i.d. 250 $\mu$m) would suffer almost no loss in peak capacity due to inefficient sample transfer. In addition, two different chip designs were studied, one of which having channel distances feasible to realize using the fabrication technology that is currently available in our facility. A prototype device was built, using CNC micromilling, that could potentially achieve a maximum total peak capacity of 32,600 in only 44 minutes after the implementation of the proposed separation modes. By using high-end prototyping technology, this device could be improved and theoretically achieve peak capacity values of approximately 54,300 in 53 minutes of total analysis time.

7.2. On-going work and future perspectives

One of the most important topics and the main one that was not treated experimentally in the present thesis is detection. In “Section 4.3.4” the hyphenation with MALDI MS imaging has been proposed as the most feasible solution to the detection issue, being able to maintain the characteristics of the already spatially resolved analytes eluting out of the chip. To this scope initial work has been performed on an in-house built robotic three-dimensional translation stage. Precision stages integrated with two-phase micro-stepper motors that can provide resolution of 5 $\mu$m and loading capacity of 10 kg in the X-Y dimension as well as resolution of 2 $\mu$m and loading capacity of 4 kg in the Z dimension, were purchased (GMT
Europe GmbH, Germany). The 3-axis stage was programmed using XIMC software to precisely position and move the MALDI plate towards the chip placed in a fixed position. Additionally, a tilt-stage was acquired (GMT Europe GmbH, Germany) mounted on the Z-axis motorized stage. This serves as a positioning place for the stainless steel MALDI plate, and its angle can be regulated with a precision of 0° 15’ per manual rotation, in order to permit the exact alignment of the plate and the outlets of the spatial chip. A polished stainless-steel plate of 50×50 mm was initially used as target plate for the deposition of the droplets coming out of a 3D printed chip that has a channel layout of a 3D flow distributor with tapered outlets and channel diameter of 200 μm (Fig 7.1).

The deposited droplets were all round shaped but with non-uniform size having a mean size of 2.93 mm² but a large RSD of 31% after measurement using an USB optical microscope. Large sized spots can lead to reduced MS intensities as the laser spot of 200 μm diameter will target only a specific very small zone of the droplet. This large dimension of the spots can be attributed to the fact that the droplets spread on the stainless steel surface of the MALDI target plate. Additionally, our setup involves a moving MALDI plate and a fixed separation device. This movement can lead to further irregularity of the spot size. For this reason, a series of modifications must be introduced. First, the chip should be mounted on the 3D translation stage while the MALDI target plate must have a fixed position. Moreover, another two solutions are going to be pursued in our coming attempts. The first is the formation (via micromilling) of micro-sized wells on the target plate that could accommodate the effluent droplets and physically block the spreading. The second one is spin-coating of the stainless steel surface with a thin layer highly hydrophobic agent i.e., Teflon and baking it at a high temperature to produce the stable thin layer. In this way the hydrophobicity of the surface is going to increase.
yielding rounder and more regular spots. Testing of the spotting efficiency must be performed and data should be acquired as a function of flow rate and of the deposition frequency. At a first stage, mobile phase spiked with FITC is going to be initially used for the above mentioned experiments due to the high sensitivity that this can provide with fluorescence microscopy. Following, that a simple separation of a mixture of proteins will be performed in a device containing 8 parallel channels and the spotting efficiency is going to be evaluated through the acquisition of MALDI MS data. Assessing the spot size and shape as a function of the spotting frequency is fundamental for a spatial MD-LC separation. Theoretical studies using Pareto optimization have shown that increasing the number of the 2D and 3D channels can lead not only to higher total peak capacity for a spatial 3D-LC device but also to increased peak-production rates ($\xi$) as demonstrated in Table 7.1. As a consequence, higher peak-production rates require higher frequency of stamp collection on the surface of the MALDI plate in order to be able to maintain the resolving power obtained by the preceding spatial 3D-LC separation. This stamping frequency can be mainly limited by two factors. One is the mechanical properties of the translation stage motors which is an inherent characteristic of the device. The other is a combination of the flow rate applied in the chip together with the clearing distance of the chip from the MALDI plate. These two parameters should be fine-tuned proportionally to each other in order to be able to achieve optimal spotting quality at higher frequencies.

When it comes to the actual MS analysis, after the deposition of the effluent of the 3D on the plate there are different approaches that can be followed. The classic top-down proteomics studies that have been largely benefited by the introduction of the Orbitrap Fusion Lumos mass analyzer and especially its implementation with higher capacity electron-transfer dissociation fragmentation mode can be a well-established approach [1–3]. Nevertheless, the $m/z$ range that needs to be scanned for intact protein ions in a global proteomics experiment can be very wide and this can be a limiting factor when it comes to protein identification. Fortunately, the nature of a spatial 3D-LC separation can provide crucial input in this case and assist the identification process. Having a charge-based 1D separation (IEF) combined with a size based 2D separation (SEC) can give information leading to reduced ranges of $m/z$ for each spot based on its respective position in the X-Y plans. To get though such information, significant work should be carried out on a software that could simulate a spatial 2D-LC, 1IEF × 2SEC separation and give the final positions of the expected charge and molecular weight coordinates in the X-Y plane from which the needed range of $m/z$ to be scanned can be calculated.
Table 7.1. Optimal combinations of $L$, $\alpha$ (distance between the channels) and $d_p$ for a spatial 3D-LC device integrating 1IEF $\times$ 1SEC $\times$ RPLC with the goal to reach peak capacity values of 2500, 10000, 25000, 50000 for the separation of intact proteins.

<table>
<thead>
<tr>
<th>$n_c$</th>
<th>1D-IEF</th>
<th>2D-SEC</th>
<th>3D-RP</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$L$ (cm)</td>
<td>$\alpha$ (cm)</td>
<td>$L$ (cm)</td>
<td>$\alpha$ (cm)</td>
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<tr>
<td>2500</td>
<td></td>
<td></td>
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<tr>
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<td>0.005</td>
<td>1.40</td>
<td>0.005</td>
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<tr>
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<td>0.005</td>
<td>3.00</td>
<td>0.005</td>
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<td>0.04</td>
<td>2.20</td>
<td>0.09</td>
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<td>1.60</td>
<td>0.005</td>
<td>3.20</td>
<td>0.005</td>
<td>320</td>
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## CONCLUSIONS, ON-GOING WORK, AND FUTURE PERSPECTIVES

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<tr>
<th></th>
<th>( n = 10000 )</th>
<th>( n = 25000 )</th>
<th>( n = 50000 )</th>
</tr>
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<tr>
<td></td>
<td>( \alpha = 0.4-1.5 \text{ mm} )</td>
<td>( \alpha = 0.05-0.4 \text{ mm} )</td>
<td>( \alpha = 0.05-0.4 \text{ mm} )</td>
</tr>
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<td></td>
</tr>
<tr>
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<td>1.90 22 1.50</td>
<td>16.56 9940 600 27.3</td>
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<tr>
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<td>16.95 10034 592 9.1</td>
</tr>
<tr>
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<tr>
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<td>2.50 4 1.60</td>
<td>31.89 10026 314 78.6</td>
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<tr>
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<td>4.80 0.04 75 1.53</td>
<td>2.60 120 1.60</td>
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<tr>
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<td>4.50 0.09 78 1.51</td>
<td>2.30 50 1.50</td>
<td>21.02 24789 1179 12.4</td>
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<tr>
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<td>1.7 0.005 620 1.54</td>
<td>1.6 340 1.5</td>
<td>34.44119 25219 732 6.1</td>
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<td>4.9 0.015 620 1.55</td>
<td>1.2 327 1.6</td>
<td>34.15134 24979 731 14.6</td>
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<td>1.2 327 1.6</td>
<td>46.79475 49897 1066 3.2</td>
</tr>
<tr>
<td>( n_{c} )</td>
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It becomes intuitive to realize that these m/z ranges can be even more precise when increasing the number of channels in the spatial 3D-LC device, because shorter intervals of charges and molecular weights will be more accurately sampled from the 1D and 2D. But even when the number of channels in the 2D and 3D is not high enough to provide a limited range of m/z, other approaches can be applied to help acquire additional information that can assist protein identification. Such a novel approach involves the use of ion mobility (IM)-MS instrumentation where information on the ion size through the use of orientationally averaged collision cross sections (CCSs) can be acquired [4]. This data combined with modern machine learning techniques together with the already existing data information provided by the online protein databank can aid the identification of proteins after spatial 3D-LC analysis.

Another workflow that may be worth exploring, when it comes to MS detection of the deposited effluent coming out of the spatial 3D-LC device, is the use of nanoPOTS (Nanodroplet Processing in One Pot for Trace Samples) as an alternative target plate of the effluent of the 3D separation where in-situ digestion of the spots can take place. This will be followed by peptide MS fingerprinting at the MS1 level or peptide sequencing at the MS/MS level using state-of-the-art Orbitrap Fusion Lumos or TIMS-TOF MS analyzers [5,6].

Apart from the technical issues that need to be tackled in order to obtain proper MS spectra and identification of the spatially separated analytes, there are also issues related to the alignment of the 2D data acquired from each deposited array of spots as well as the 3D visualization of the entire separation. Software packages, used in tissue MSI, able to reconstruct such images are already available in open source format allowing to create three-dimensional representation of the data. Additionally, it must be stated that even if spatial 3D-LC has been introduced as a high-efficiency and high throughput method the employed time to perform the MS detection and acquire the data may be a limiting factor time-wise. Indicatively, a spatial 3D-LC device with a 9000 3D channels that can yield a total peak capacity of 25000 in 34 minutes (as indicated from the estimated values in Table 7.1) would require 6.1 stamps (of the 3D effluent) per minute, in order not to lose the achieved resolution during the MSI. Hence, this setup would produce 6 MALDI plates to be processed, each of them containing 9000 spots. As reported in Chapter 4, already established technology in the field of diagnostics can process 1536 spots in under 8 minutes which means that in the aforementioned case the MALDI analysis by itself would require a total of 280 minutes roughly, bringing the total analysis time to 315 minutes thus reducing the throughput of this technology. Significant work must be put on this task, in order to remove this limiting factor, proving once again that mass spectrometry
and chromatography should be moving along together towards new break-throughs in the field of analytical chemistry.

A field where considerable development has been carried out the last ten to fifteen years regarding the manufacturing process of chips is 3D printing [7]. Such rapid manufacturing methods can provide fast and inexpensive ways to create devices with interconnected channels like the ones used in spatial MD separations. Significant effort has already been put from our research group on the topic of optimizing the 3D printing parameters to create microfluidic chips with high-resolution features. Further advances in this topic can help to achieve important details such as the reduction of the distance between the channels, the number of which can greatly affect the performance of a spatial 3D-LC device as explained in “Sections 6.4.3-4”. However, high-resolution 3D printing in spatial MD-LC is not the only requirement. When attempting to perform efficient separations, the pressure resistance of the device is fundamental. On this aspect our research group has managed to build devices that can withstand backpressures as high as 200 bar and further improvement can be expected in the near future [8]. The following step that should be taken is to study the possibility of integrating arrays of microvalves in the 3D printed device in order to achieve flow confinement. The research should focus on ways to make these Quake-style microvalves more resistant to higher backpressures as until now the pressure resistance is limited to a few bar [9]. Such valves combined with tuning the permeabilities of the stationary phase in each dimension can prove to be effective means to maintain the analytes and the flow in the predetermined separation space. As an alternative, with the continuous advancing of multi-material 3D printing, multichannel devices incorporating smart materials, responsive to stimuli, in specific segments, can be envisioned as means to fabricate controllable active gates within the device with the goal to achieve flow confinement [10]. Additionally, another part that should be examined is the type of resins applied in the process of 3D printing. As mentioned earlier, high-resolution is one of the goals to achieve in 3D printing of microdevices but not the only one. Advances in the discovery of new resins with higher resistance to organic solvents is fundamental for their application in devices for liquid-phase separations given the fact the most efficient and widespread separation mode makes rather extensive use of organic solvents [11,12]. On top of that novel resin chemistries exhibiting high UV transparency and low autofluorescence should be pursued [13]. The currently used resins that yield optimal results in terms of resolution demonstrate low penetrability by the UV light, a fact that greatly limits our possibility to incorporate organic monolithic stationary phases. To circumvent this problem, channels with a serpentine structure
are printed, having the sections which are chosen to incorporate the monolithic stationary phase closer to the chip surface (3 to 4 layers distance, corresponding to 60-80 μm) which of course causes reduced resistance to backpressure in these specific channel tracts and hence forces us to make used of specific custom made holders that encase the device and improve the total pressure resistance.

7.3. References


doi:10.1021/acs.analchem.0c01551.


8.1. List of symbols and abbreviations

8.1.1. Symbols

A \quad \text{Eddy-dispersion contribution to band broadening (μm)}

a \quad \text{Reduced eddy-dispersion contribution to band broadening (l)}

B \quad \text{Longitudinal or axial diffusion contribution to band broadening (mm}^2\text{/s)}

b \quad \text{Reduced longitudinal diffusion contribution to band broadening (l)}

C \quad \text{Resistance to mass-transfer contribution to band broadening (ms)}

c \quad \text{Reduced mass-transfer contribution to band broadening (l)}

C_m \quad \text{Resistance to mobile-phase mass-transfer contribution (ms)}

C_s \quad \text{Resistance to stationary-phase mass-transfer contribution (ms)}

D_m \quad \text{Mobile-phase diffusion coefficient (m}^2\text{/s)}

D_s \quad \text{Stationary-phase diffusion coefficient (m}^2\text{/s)}

d_p \quad \text{Particle diameter (μm)}

E \quad \text{Electric field (V/m)}

F \quad \text{Flow rate (m}^3\text{/s)}

F \quad \text{Faraday constant (C/mol·e⁻)}

H \quad \text{Plate height (μm)}

h \quad \text{Reduced plate height (l)}

k \quad \text{Retention factor (l)}

k'' \quad \text{Zone retention factor (l)}

k_0 \quad \text{Retention factor at the start of the gradient (l)}

k_B \quad \text{Boltzmann constant (J/K)}

k_a \quad \text{Retention factor of the first eluting compound (l)}

k_{wa} \quad \text{Retention factor of the last eluting compound (l)}

k_G \quad \text{Gradient retention factor (l)}
$k_s$ Retention factor relative to the compound excluded from the pores (l)

$K_D$ Thermodynamic retention factor (l)

$K_{v,0}$ Column permeability (m$^2$)

$L$ Length (m)

$MW$ Molecular weight (Da)

$MW_e$ Molecular weight at the point of exclusion (Da)

$n_c$ Peak capacity (l)

$N$ Plate number (l)

$N_{KPL}$ Plate number at the kinetic performance limit (l)

$q$ Charge of a molecule (C)

$q_m$ Maximum net charge of a molecule (l)

$r$ Radius of an analyte (m)

$R$ Ideal gas constant (J/K·mol)

$R_F$ Retardation factor (l)

$R_s$ Resolution (l)

$R_{\Phi,def}$ Optimal mixing rate (l)

$S$ Linear solvent-strength parameter (l)

$T$ Temperature (K)

$t_0$ Column dead time (min)

$t_G$ Gradient time (min)

$t_i$ Retention time of a compound that does not penetrate the pores (min)

$t_R$ Retention time (min)

$t_{R,last}$ Retention time of the last eluted compound (min)

$t_s$ Sampling time (min)

$u_0$ Mobile-phase linear velocity for an analyte fully penetrating the pores (mm/s)

$u_e$ Mobile-phase linear velocity for an analyte not penetrating the pores (mm/s)
\[ \nu_{opt} \quad \text{Optimal linear velocity (mm/s)} \]
\[ \nu \quad \text{Reduced velocity (l)} \]
\[ \nu_z \quad \text{Reduced interstitial velocity (l)} \]
\[ \nu_E \quad \text{Electrophoretic velocity (m/s)} \]
\[ V_i \quad \text{Intraparticle volume (\(\mu\text{L}\))} \]
\[ V_D \quad \text{Interstitial volume (\(\mu\text{L}\))} \]
\[ V_R \quad \text{Retention volume (\(\mu\text{L}\))} \]
\[ W \quad \text{Peak width (min)} \]

8.1.2. Greek symbols

\[ \alpha \quad \text{Selectivity (l)} \]
\[ \beta \quad \text{Undersampling factor (l)} \]
\[ \gamma \quad \text{Obstruction factor (l)} \]
\[ \Delta c \quad \text{Gradient span (l)} \]
\[ \Delta P \quad \text{Pressure drop (bar)} \]
\[ \epsilon \quad \text{Dielectric constant (F/m)} \]
\[ \epsilon_e \quad \text{External porosity (l)} \]
\[ \epsilon_t \quad \text{Total porosity (l)} \]
\[ \zeta \quad \text{Zeta potential (V)} \]
\[ \eta \quad \text{Solvent dynamic viscosity (Pa\cdot s)} \]
\[ \lambda \quad \text{Column elongation factor (l)} \]
\[ \lambda_{bed} \quad \text{Bed tortuosity (l)} \]
\[ \mu_E \quad \text{Electrophoretic mobility (m}^2/V\cdot s) \]
\[ \mu_{EOF} \quad \text{Electro-osmotic mobility (m}^2/V\cdot s) \]
\[ \xi \quad \text{Peak production rate (l)} \]
\[ \sigma \quad \text{Standard deviation (l)} \]
\[ \sigma_t^2 \quad \text{Time variance of a chromatographic peak (s}^2) \]
\[ \sigma_x^2 \quad \text{Spatial variance of a chromatographic peak (m}^2) \]
\[ \sigma_v^2 \quad \text{Volumetric variance of a chromatographic peak (}\mu l^2) \]
\[ \phi \quad \text{Column flow resistance (l)} \]
\[ \omega \quad \text{Gradient occupancy (l)} \]

### 8.1.3. Abbreviations

1D  Unidimensional

1D  First dimension

2D  Two-dimensional

2D  Second dimension

3D  Three-dimensional

3D  Third dimension

MD  Multi-dimensional

ACN  Acetonitrile

BMA  Butyl methacrylate

BP  Benzophenone

BSA  Bovine serum albumin

C\(_4\)  Aliphatic chain with 4 carbons

C\(_{18}\)  Aliphatic chain with 18 carbons

CE  Capillary electrophoresis

CEC  Capillary electrochromatography

CGE  Capillary gel electrophoresis

COC  Cyclic olefin copolymer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>COMOSS</td>
<td>Collocated monolithic support structures</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMPA</td>
<td>2,2 dimethoxy-2-phenylacetophenone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDMA</td>
<td>Ethylene dimethacrylate</td>
</tr>
<tr>
<td>EGDA</td>
<td>Ethylene glycol diacrylate</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
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<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>i.d.</td>
<td>Internal diameter</td>
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<td>IEF</td>
<td>Isoelectric focusing</td>
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<td>IEX</td>
<td>Ion-exchange chromatography</td>
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<tr>
<td>KPL</td>
<td>Kinetic performance limit</td>
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<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC\times LC</td>
<td>Online comprehensive 2D-LC</td>
</tr>
<tr>
<td>LC/\times LC</td>
<td>Offline 2D-LC</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MD</td>
<td>Multidimensional</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar electrokinetic chromatography</td>
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<tr>
<td>MMA</td>
<td>Methyl methacrylate</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>MS/MS</td>
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<td>Abbr.</td>
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</tr>
<tr>
<td>NP</td>
<td>Normal-phase chromatography</td>
</tr>
<tr>
<td>OPTLC</td>
<td>Overpressured thin-layer chromatography</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDMS</td>
<td>Polymethylsiloxane</td>
</tr>
<tr>
<td>PEEK</td>
<td>Poly(ether ether ketone)</td>
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<td>PFA</td>
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<tr>
<td>RP</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SEC</td>
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<td>TOF</td>
<td>Time of flight</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
8.2. List of publications

*Engineering solutions for flow control in microfluidic devices for spatial multi-dimensional liquid chromatography*
T. Themelis, J. De Vos, J. L. Dores-Sousa, T. van Assche, S. Eeltink
Sensors & Actuators: B. Chemical 320 (2020) 12838

*Towards spatial comprehensive three-dimensional liquid chromatography: a tutorial review*
T. Themelis, A. Amini, J. De Vos, S. Eeltink
Analytica Chimica Acta 1148 (2021) 238157

*Design guidelines and kinetic performance limits for spatial comprehensive three-dimensional chromatography for the analysis of intact proteins*
T. Themelis, J. De Vos, S. Eeltink
Analytical Chemistry 2022, 94, 13737−13744

*Development of a generic ultra-high-pressure gradient liquid-chromatography method development protocol: The analysis of residual multi-class antibiotics in food products as a case study*
Journal of Chromatography A 1684 (2022) 463565

*Recent developments in digital light processing 3D-printing techniques for microfluidic analytical devices*
A. Amini, R. M. Guijt, T. Themelis, J. De Vos, S. Eeltink
Journal of Chromatography A (revisions requested)
8.3. Authors’ contribution

CHAPTER 1

SCOPE AND OUTLINE

Thomas Themelis: Wrote the chapter
Sebastiaan Eeltink: Reviewed the chapter

CHAPTER 2

INTRODUCTION TO THE BASIC CONCEPTS OF ONE- AND MULTI-DIMENSIONAL SEPARATIONS

Thomas Themelis: Wrote the chapter
Sebastiaan Eeltink: Reviewed the chapter

CHAPTER 3

DEVELOPMENT OF A GENERIC ULTRA-HIGH-PRESSURE GRADIENT LIQUID-CHROMATOGRAPHY METHODOLOGY FOR THE ANALYSIS ANTIBIOTICS IN FOOD PRODUCTS

Daniel Meston: Data collection, analysis and interpretation of results, writing
Thomas Themelis: Data collection, analysis and interpretation of results, writing
Zhuoheng Zhou: Analysis and interpretation results, writing
Jelle De Vos: Reviewing
Mauro De Pra: Conceptualization and reviewing
Frank Steiner: Reviewing
Ilse Becue: Providing samples
Els Daeseleire: Providing samples and reviewing
Gert Desmet: Reviewing, funding acquisition
Sebastiaan Eeltink: Reviewed the chapter
CHAPTER 4

TOWARDS SPATIAL COMPREHENSIVE THREE-DIMENSIONAL LIQUID CHROMATOGRAPHY

Thomas Themelis: Wrote the chapter
Ali Amini: Wrote the chapter
Jelle De Vos: Wrote the chapter, funding acquisition
Sebastiaan Eeltink: Conceptualization, wrote and reviewed the chapter, funding acquisition

CHAPTER 5

ENGINEERING SOLUTIONS FOR FLOW CONTROL IN MICROFLUIDIC DEVICES FOR SPATIAL MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY

Thomas Themelis: Conceptualization, methodology, validation, wrote the chapter
José L. Dores-Sousa: Methodology
Jelle De Vos: Conceptualization, supervision, funding acquisition
Tom van Assche: Methodology, reviewing
Sebastiaan Eeltink: Reviewed the chapter, supervision, funding acquisition

CHAPTER 6

DESIGN GUIDELINES AND KINETIC PERFORMANCE LIMITS FOR SPATIAL COMPREHENSIVE THREE-DIMENSIONAL CHROMATOGRAPHY FOR THE ANALYSIS OF INTACT PROTEINS

Thomas Themelis: Conceptualization, experiments, wrote the chapter
Jelle De Vos: Review, funding acquisition
Sebastiaan Eeltink: Reviewed the chapter, supervision, funding acquisition
CHAPTER 7

CONCLUSIONS, ON-GOING WORK, AND FUTURE PERSPECTIVES

Thomas Themelis: Wrote the chapter
Sebastiaan Eeltink: Reviewed the chapter
8.4. MATLAB scripts

8.4.1. Calculation of spatial 3D-LC Pareto fronts

```matlab
% FlatBed=1; % This must be turned on when flat bed is simulated
FlatBed=0; % This must be turned on when discrete channel design is chosen

channel_spacing=1; % Turn on this for channel distances ranging between 0.05 mm - 0.4 mm
channel_spacing=2; % Turn on this for channel distances ranging between 0.4 mm - 1.5 mm
channel_spacing=3; % Turn on this for channel distances ranging between 1.5 mm - 15 mm

if channel_spacing==1
    dist_IEF=(0.005:0.001:0.04).*1e-2; % values in cm (0.05 mm - 0.4 mm) sampling freq distances
    dist_SEC=(0.00005:0.0001:0.0004).*1e+6; % sampling frequency in um (translates to 0.05-0.4 mm distances)
    text_dist='0.05-0.4 mm';
end

if channel_spacing==2
    dist_IEF=(0.04:0.001:0.15).*1e-2; % values in cm (0.4 mm -1.5 mm) sampling frequency distances
    dist_SEC=(0.0004:0.0005:0.0015).*1e+6; % sampling frequency in um (translates to 0.4-1.5 mm distances)
    text_dist='0.4-1.5 mm';
end

if channel_spacing==3
    dist_IEF=(0.15:0.001:1.5).*1e-2; % values in cm (1.5 mm -15 mm) sampling frequency distances
    dist_SEC=(0.0015:0.0005:0.015).*1e+6; % sampling frequency in um (translates to 1.5-15 mm distances)
    text_dist='1.5-15 mm';
end

% ---------------- Common parameters --------------------------
```
P=2e+7; % pressure of the device in Pascal (converted in N/um^2) FOR ALL DIMENSIONS
v=0.001; % viscosity of mobile phase in Pascal * s FOR ALL DIMENSIONS

%-----------------1st dimension IEF parameters-----------------------------

E=500; % Field (Volts/cm)
F=96485.3329; % Joule/volt (Faraday constant)
dq_dpH=-9; % For ovalbumin
L_IEF=(1:0.1:5)*1e-2; % Length of the device in cm
Delta_pH=7; % Difference between pH at the end of the column and the
begining of the column
R=8.314472; % Ideal gas constant
T=298; % Temperature [K]
q=20; % Is the max net charge that the protein can assume in the pH range
of the channel
Dm_IEF=2.6e-11; % for ovalbumin in m^2/s
K=8.617e-5; % Boltzman constant [Joules K]

%-----------------2nd dimension SEC parameters-----------------------------

L_SEC=(0.01:0.001:0.05)*1e+6; % Length of the column (um)
dp_SEC=(1.5:0.01:5); % Particle size (um)
phi_SEC=400;
tot_por=0.7;
ext_por=0.44;
Dm_SEC=260; % um^2/s diffusion coeff of large protein 900 kDa
Vi_V0_ratio=1.2043;
Y=-5; %slope of the linear part of the calibration curve
m=-Vi_V0_ratio*(1/Y);
MW_mr=1350; %MW smallest analyte. Better option to use the MW of Vitamin
B12 to represent the full range of retention volumes
MWe=1e+6; %MW at the point of exclusion

% Reduced Van Deemter parameters for SEC (values from Vajda et al JCA 2015)
a_SEC=2;
b_SEC=2;
c_SEC=0.6;
% 3rd dimension RP parameters

L_RP=(0.01:0.001:0.05); % Length of the column (meters)
Delta_c=0.3; % Mobile phase composition (variation of organic modifier)
S=70; % Slope of lnk versus phi
dp_RP=(1.5:0.1:5).*1e-6; % Particle size (m)
phi_RP=650; %value from Desmet TrAC 2014
tg_t0_ratio=20;
Dm_RP=1e-11; % diffusion coefficient for large molecules

% Reduced Van Deemter parameters for RP (values from GVT Anal. Chem. 2010)
a_RP=1.5; %values from GVT Anal. Chem.
b_RP=1;
c_RP=0.15;

% Calculations

[n_1D_IEF_mod,t_1D_IEF_mod,L_IEF,dist_IEF]=IEF_1D_for_spatial_sampling(L_IEF,dist_IEF,F,E,dq_dpH,Delta_pH,R,T,q,Dm_IEF,K,FlatBed);
[n_2D_SEC_mod,t_2D_SEC_mod,L_SEC,dp_SEC,dist_SEC]=SEC_2D_for_spatial_sampling(L_SEC,dp_SEC,dist_SEC,m,MW_mr,MWe,phi_SEC,F,v,tot_por,ext_por,Dm_SEC,a_SEC,b_SEC,c_SEC,FlatBed);
[n_3D_RP_f,t_3D_RP_f,L_RP,dp_RP]=RP_3D_for_spatial_sampling(L_RP,dp_RP,P,Delta_c,S,phi_RP,v,tg_t0_ratio,Dm_RP,a_RP,b_RP,c_RP);

PC_2D_mod=n_1D_IEF_mod.*n_2D_SEC_mod'; %product of 1D and 2D !!!ATTENTION
the transpose vector of n_2D_SEC_mod must be used

PC_2D_mod=PC_2D_mod(:,); % transforming the matrixes in column vectors

PC_3D_spatial=PC_2D_mod.*n_3D_RP_f'; %Total peak capacity !!!ATTENTION the
transpose vector of n_3D_RP_f must be used

PC_3D_spatial=PC_2D_mod.*t_3D_RP_f'; %Total analysis times !!!ATTENTION the
transpose vector of t_3D_RP_f must be used
PC_3D_spatial=PC_3D_spatial(:); % transforming the matrixes in column vectors
t_3D_spatial=t_3D_spatial(:);

fnd=Pareto([PC_3D_spatial t_3D_spatial*(-1)]);
ParetoExperiment=find(fnd==1);

filename=['IEFxSECxRP revisions 0.5 red ',text_dist,'.xlsx'];
xlswrite(filename,PC_3D_spatial(ParetoExperiment),'Pareto front','A2');
xlswrite(filename,t_3D_spatial(ParetoExperiment)./60,'Pareto front','B2');

%------------------Plots------------------------------------------
figure(1)
plot(PC_3D_spatial(ParetoExperiment),t_3D_spatial(ParetoExperiment)./60,'o'),xlabel('Peak capacity'), ylabel('t_ total_ (min)')

8.4.2. Pareto front in 1D-IEF

function

[n_1D_IEF_mod,t_1D_IEF_mod,L_IEF,dist_IEF]=IEF_1D_for_spatial_sampling(L_IEF,dist_IEF,F,E,dq_dpH,Delta_pH,R,T,q,Dm_IEF,K,FlatBed)

L_IEF_0=repmat(L_IEF,1,numel(dist_IEF));L_IEF_0=L_IEF_0(:);
dist_0=repmat(dist_IEF,numel(L_IEF),1);dist_0=dist_0(:);

dist_IEF=dist_0; clear dist_0
L_IEF=L_IEF_0; clear L_IEF_0

s_IEF=L_IEF./(4.*n_1D_IEF); % Sigma of the band
t_1D_IEF=L_IEF.*K.*T./(q*Dm_IEF*E); % Electrophoresis, 2003, 24 3735-3744, Eq. (14)

if FlatBed==0
    n_1D_IEF_m=zeros(length(dist_IEF),1);

for i=1:length(dist_IEF)
    if dist_IEF(i,1)>=4*s_IEF(i,1)

\[ n_{1D\_IEF\_m}(i,1) = \left( \frac{L_{IEF}(i,1)}{dist\_IEF(i,1)} \right) \times (1 - 2 \times s_{IEF}(i,1)/dist\_IEF(i,1)); \] % Correction of peak capacity due to undersampling. Careful: sigma << a. Otherwise, use \( L/4\times\sqrt{\sigma^2 + a^2/4.76} \)

else

\[ n_{1D\_IEF\_m}(i,1) = \frac{L_{IEF}(i,1)}{4\times\sqrt{s_{IEF}(i,1)^2 + (dist\_IEF(i,1)^2/4.76)}}; \] % Correction of peak capacity in case of \( \sigma \geq a \) (here dist)

end
end

\[ s_{IEF\_m} = \frac{L_{IEF}}{4\times n_{1D\_IEF\_m}}; \] % Sigma of the band after modulation

\[ ch\_num = (L_{IEF}/dist\_IEF) - 1; \] % Number of channels

\[ s_{norm} = \frac{s_{IEF}}{s_{IEF\_m}}; \]

\[ n_{IEF\_norm} = \frac{n_{1D\_IEF\_m}}{n_{1D\_IEF}}; \]

\[ j = 1; \]

for \( i = 1:\text{length}(n_{IEF\_norm}) \)

if \( n_{IEF\_norm}(i,1) \geq 0 \)

\[ n_{IEF\_norm\_f}(j,1) = n_{IEF\_norm}(i,1); \]

\[ ch\_num\_IEF\_f(j,1) = ch\_num(i,1); \]

\[ j = j + 1; \]

end
end

\[ fnd = \text{Pareto}([n_{1D\_IEF\_m} t_{1D\_IEF}\_m*(-1)]); \]

\[ \text{ParetoExperiment} = \text{find}(fnd==1); \]

\[ n_{1D\_IEF\_mod} = n_{1D\_IEF\_m}(\text{ParetoExperiment}); \] % Creating vectors with the Pareto optimal values for discrete channel design to save

\[ t_{1D\_IEF\_mod} = t_{1D\_IEF}(\text{ParetoExperiment}); \]

else

\[ fnd = \text{Pareto}([n_{1D\_IEF} t_{1D\_IEF}\_m*(-1)]); \]

\[ \text{ParetoExperiment} = \text{find}(fnd==1); \]

\[ n_{1D\_IEF\_mod} = n_{1D\_IEF}(\text{ParetoExperiment}); \] % Creating vectors with the Pareto optimal values for the flat bed design to save

\[ t_{1D\_IEF\_mod} = t_{1D\_IEF}(\text{ParetoExperiment}); \]
8.4.3. Pareto front in 2D-SEC

function
[n_2D_SEC_mod,t_2D_SEC_mod,L_SEC,dp_SEC,dist_SEC]=SEC_2D_for_spatial_sampling(L_SEC,dp_SEC,dist_SEC,m,MW_mr,MWe,phi_SEC,P,v,tot_por,ext_por,Dm_SEC,a_SEC,b_SEC,c_SEC,FlatBed)

L_0=repmat(L_SEC,1,numel(dp_SEC),numel(dist_SEC));L_0=L_0(:);
dp_0=repmat(dp_SEC,numel(L_SEC),1,numel(dist_SEC));dp_0=dp_0(:);
dist_0=repmat(dist_SEC,numel(L_SEC),numel(dp_SEC),1);dist_0=dist_0(:);

L_SEC=L_0; clear L_0
dp_SEC=dp_0; clear dp_0
dist_SEC=dist_0; clear dist_0

ks=m.*log10(MWe./MW_mr); %retention factor
ks=abs(ks);
Rf=1/(1+ks);

% Time / peak capacity
u0=P.*(dp_SEC.^2)./(phi_SEC.*v.*L_SEC); % Darcy equation
ui=(u0.*tot_por)./ext_por; %calculation of the interstitial velocity according to Desmet paper
t0=L_SEC./ui; % Dead time
uiired=ui.*dp_SEC./Dm_SEC; % Reduced interstitial velocity

% Van Deemter
hred=a_SEC+(b_SEC./(Rf.*uiired))+c_SEC.*(1-Rf)^2.*uiired;

H=hred.*dp_SEC; % From reduced plate height to absolute plate height
N=L_SEC./H; % Number of plates

n_2D_SEC=1+0.25*sqrt(N).*(log(1./Rf)); %peak capacity for development
s_2D_SEC=L_SEC./(4.*n_2D_SEC); % Sigma of the band
t_2D_SEC=t0; % This is the total analysis time
if FlatBed==0

n_2D_SEC_m=zeros(length(dist_SEC),1);

for i=1:length(dist_SEC)
    if dist_SEC(i,1)>=4*s_2D_SEC(i,1)
        n_2D_SEC_m(i,1)=(L_SEC(i,1)./dist_SEC(i,1)).*(1-2.*s_2D_SEC(i,1)./dist_SEC(i,1)); % Correction of peak capacity due to undersampling for sigma << dist.
    else
        n_2D_SEC_m(i,1)=L_SEC(i,1)./(4.*sqrt(s_2D_SEC(i,1).^2 + (dist_SEC(i,1).^2)./4.76))); % Correction of peak capacity in case of sigma>=dist
    end
end

s_2D_SEC_m=L_SEC./(4.*n_2D_SEC_m); % Sigma of the band after modulation

ch_num=(L_SEC./dist_SEC)-1; %number of channels
s_norm=s_2D_SEC./s_2D_SEC_m;

j=1;

for i=1:size(s_norm,1)
    if s_norm(i,1)>=0
        s_norm_f(j,1)=s_norm(i,1);
        ch_num_SEC_f(j,1)=ch_num(i,1);
        j=j+1;
    end
end

fnd=Pareto([n_2D_SEC_m t_2D_SEC*(-1)]);
ParetoExperiment=find(fnd==1);
n_2D_SEC_mod=n_2D_SEC_m(ParetoExperiment); %creating vectors with the Pareto optimal values for discrete channel design to save t_2D_SEC_mod=t_2D_SEC(ParetoExperiment);

else
fnd=Pareto([n_2D_SEC t_2D_SEC*(-1)]);
ParetoExperiment=find(fnd==1);
n_2D_SEC_mod=n_2D_SEC(ParetoExperiment); %creating vectors with the Pareto optimal values for the flat bed design to save
t_2D_SEC_mod=t_2D_SEC(ParetoExperiment);
ch_num_SEC_f=0;
end
end

8.4.4. Pareto front in 3D-RP

function
[n_3D_RP_f,t_3D_RP_f,L_RP,dp_RP]=RP_3D_for_spatial_sampling(L_RP,dp_RP,P,De lta_c,S,phi_RP,v,tg_t0_ratio,Dm_RP,a_RP,b_RP,c_RP)

L_0=repmat(L_RP,1,numel(dp_RP));L_0=L_0(:);
dp_0=repmat(dp_RP,numel(L_RP),1);dp_0=dp_0(:);

L_RP=L_0; clear L_0
dp_RP=dp_0; clear dp_0

% Time / peak capacity
u=P*(dp_RP.^2)./(phi_RP.*v.*L_RP); % Darcy equation
tm=L_RP./u; % Dead time
ured=u.*dp_RP./Dm_RP; % Reduced velocity
%uopt=sqrt(b./c);

% Van Deemter
hred=a_RP+b_RP./ured+c_RP.*ured;
H=hred.*dp_RP; % From reduced plate height to absolute plate height

N=L_RP./H; % Number of plates
n_3D_RP=1+((sqrt(N)/4)*S*Delta_c)./((S*Delta_c./tg_t0_ratio)+1);
tg_RP=tm.*(tg_t0_ratio); % this is the gradient time
t_3D_RP=tm+tg_RP; % This is the total analysis time

fnd=Pareto([n_3D_RP t_3D_RP*(-1)]);
ParetoExperiment=find(fnd==1);
n_3D_RP_f=n_3D_RP(ParetoExperiment); %creating vectors with the Pareto optimal values to save
t_3D_RP_f=t_3D_RP(ParetoExperiment);
end
8.5. Acknowledgements

So, this is it! Four years and a pandemic later, what did I get from this journey? Well, a title, some friends, and the umpteenth confirmation that I like doing research not only when results come easy but also when things take unexpected turns.

There are many people that helped me during these four years and to whom I owe my sincere gratitude, but my first words must go to my promotor Prof. Dr. Sebastiaan Eeltink. Bas, thank you very much for giving me the opportunity to be part of your research group, for trusting me with this task and for the input that you provided during this period. Special thanks are also due to Dr. Jelle De Vos, my co-promotor and the person that showed me all the tips and tricks in the lab (and there were a lot) and helped me with a lot of scientific discussions. I am also deeply grateful to Dr. Gabriel Vivó-Truyols for the time that he spent teaching me what turned out to be a big part of the basis of my scientific research. Your genuine love for science generally and your pure approach on research became really inspiring for me. My appreciation goes to the members of my PhD jury committee Prof. Dr. Ann Van Eeckhaut, Prof. Dr. Ir. Iris De Graeve, Prof. Dr. Marianne Fillet, Dr. Ir. Jeroen Billen and two of my scientific heroes Prof. Dr. Ir. Gert Desmet and Prof. Dr. Michael Breadmore for taking the time to review my manuscript and for the insightful comments and revisions provided that increased the scientific quality of this thesis. A big thank you goes to all the members of CHIS and especially Sarah and Bart Degreel, Marc De Brabanter and Stijn De Belder for helping me with administrative and technical issues. These four years would not have been so beautiful without a few people that made my everyday routine so much better, and I cannot start with anyone else than José. Thanks very much for helping me go through my first months smoothly, for all the great scientific discussions that we had, but even more though for being such a good friend! Raphael, Zhuoheng, Ali and Daniel thank you friends, not only for your help and the countless hours of discussions on our topics but mostly for all the laughs, crazy moments, bad lunches, travelling experiences and a considerable amount of beers that we shared together! Stan, Markus and Dong you rock, and I wish all the best to you guys.

A special place in my heart during these years is reserved to the “Flagey mafia” group and particularly to Ravi and Agata with whom I shared many good and bad moments and even more drinks that helped us celebrate the good moments and go past the bad ones. Thank you very much guys for being my friends and I am sure we are going to have more of this in the future!
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I would have never done a PhD and certainly this book would have never been compiled if it weren’t for Professor Dr. Roberto Gotti who has been my “scientific father” since my master thesis at the University of Bologna. Prof. thank you for all the things you taught me and for transmitting to me your integrity and passion for research!

Last but most important, my family! Στα αδερφιά μου, τα από επιλογή όχι εξ’ άματος, Γιάννη, Μήτσο Μ., Ντέρτι, Μάικ, Κρις, Σταύρο, Νάπο, Μήτσο Κ., Αγγελάρα, Γεωργία, σας ευχαριστώ που με κάνατε παντα να νιώθω κοντά σας παρά το γεγονός ότι είμαι μικρά τόσα χρόνια. Στη μητέρα μου, τον πατέρα μου, την αδερφή μου και στη Θάλεια και τον Γιάννη ένα τεράστιο ευχαριστώ για όλα αυτά που έχετε κανει για eména και για την στήριξη και την υπομονή που δείχνεστε αυτά τα τέσσερα χρόνια. Σας ευχαριστώ που μου δώσατε την δυνατότητα να κάνω αυτή την διαδρομή και που κάνατε τις μουντές μέρες της Βρυξέλλας να μοιάζουν πιο ομορφες! Θα μπορούσα να γράψω ένα ξεχωριστό βιβλίο με τους λόγους για τους οποίους σας είμαι ευγνώμων και πάλι να μην είναι αρκετό... Σας αγαπώ πολύ!
8.6. Summary

This PhD research targets the design and development of one- and multi-dimensional separation strategies aiming to significantly enhance the resolving power. The kinetic performance limit of unidimensional ultra-high-pressure liquid chromatography (1D-UHPLC), operated in gradient-elution mode, has been systematically assessed targeting the analysis of sample mixtures containing over 60 antibiotics and their degradation products. Peak capacities up to 400 have been reached in approximately 2.5 hours, using a coupled-column system packed with 1.5 μm core-shell particles operated at optimized gradient steepness and at the maximum available pressure.

To significantly enhance the resolving power, a novel concept, i.e., spatial multi-dimensional chromatography (MD-LC), has been explored. Here, analytes are separated by migrating to different positions in a two (2D)- or three-dimensional (3D) separation space. Spatial MD-LC can potentially offer unprecedented resolving power in terms of peak capacity and analysis time. A Pareto optimization has been carried out considering the implementation of separation modes suitable for the analysis of intact proteins, allowing to optimize the channel design and number as well as the operating conditions. Microfluidic devices with interconnected multi-channel layouts have been prototyped. Novel approaches for flow control and confinement have been explored including the use of physical barriers, and implementation of on-chip active valving. The integration of monolithic stationary phases localized in-situ in microchannels has been demonstrated. Moreover, a prototype interface to mass-spectrometry imaging has been proposed.
Samenvatting

Dit doctoraatsonderzoek focust op het ontwerpen en de ontwikkelen van één- en multidimensionale scheidingsmethoden, met de bedoeling om de scheidingscapaciteit aanzienlijk te verhogen. De kinetische scheidingslimiet van eendimensionale ultrahoge druk vloeistofchromatografie (1D-UHPLC) in gradiënt-elutie modus is systematisch onderzocht door middel van de analyse van stalen met meer dan 60 verschillende antibiotica en hun afbraakproducten. In ongeveer 2,5 uur zijn piek-capaciteiten tot 400 bereikt met behulp van een gekoppeld kolom-systeem, gevuld met 1,5 µm core-shell-partikels en uitgevoerd bij de optimale gradiënt en maximaal beschikbare druk.

Een nieuw concept, i.e., ruimtelijke multi-dimensionale chromatografie (MD-LC), is onderzocht met de bedoeling om de scheidingscapaciteit aanzienlijk te verhogen. In ruimtelijke MD-LC worden analieten gescheiden doordat ze naar verschillende posities in een twee (2D)- of drie (3D)-dimensionele scheidingsruimte migreren. Ruimtelijke MD-LC zou potentieel een ongekend scheidingsvermogen bieden zowel aangaande de piekcapaciteit als de analysetijd. Een Pareto-optimalisatie is uitgevoerd met het oog op de implementatie van scheidingsmethodes geschikt voor de analyse van intacte eiwitten, waardoor het ontwerp van de kanalen, het aantal kanalen en de scheidingsmethode geoptimaliseerd konden worden.

Verder zijn prototypes van microfluiddische apparaten met onderling verbonden kanalen ontworpen. Nieuwe manieren voor de controle van vloeistofstroom en vloeistofbegrenzing zijn onderzocht, met inbegrip van het gebruik van fysieke barrières en de implementatie van on-chip actieve kleppen. De in situ integratie van monolieten als stationaire fase in microkanalen is aangetoond. Bovendien is er een prototype interface voor de koppeling aan massaspectrometrie voorgesteld.
SUMMARY

This PhD research targets the design and development of one- and multi-dimensional separation strategies aiming to significantly enhance the resolving power. The kinetic performance limit of unidimensional ultra-high-pressure liquid chromatography (1D-UHPLC), operated in gradient-elution mode, has been systematically assessed targeting the analysis of sample mixtures containing over 60 antibiotics and their degradation products. Peak capacities up to 400 have been reached in approximately 2.5 hours, using a coupled-column system packed with 1.5 µm core-shell particles operated at optimized gradient steepness and at the maximum available pressure.

To significantly enhance the resolving power, a novel concept, i.e., spatial multi-dimensional chromatography (MD-LC), has been explored. Here, analytes are separated by migrating to different positions in a two (2D)- or three-dimensional (3D) separation space. Spatial MD-LC can potentially offer unprecedented resolving power in terms of peak capacity and analysis time. A Pareto optimization has been carried out considering the implementation of separation modes suitable for the analysis of intact proteins, allowing to optimize the channel design and number as well as the operating conditions. Microfluidic devices with interconnected multi-channel layouts have been prototyped. Novel approaches for flow control and confinement have been explored including the use of physical barriers, and implementation of on-chip active valving. The integration of monolithic stationary phases localized in-situ in microchannels has been demonstrated. Moreover, a prototype interface to mass-spectrometry imaging has been proposed.