Fundamental Investigations of Supercritical Fluid Chromatography

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DISSERTATION

Karlstad University Studies | 2015:45

urn:nbn:se:kau:diva-37913

ISSN 1403-8099


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Distribution:
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Print: Universitetstryckeriet, Karlstad 2015
Abstract

This thesis aims at a deeper understanding of Supercritical Fluid Chromatography (SFC). Although preparative SFC has started to replace Liquid Chromatography (LC) in the pharmaceutical industry - because of its advantages in speed and its less environmental impact - fundamental understanding is still lacking. Therefore, there is no rigid framework to characterize adsorption or to understand the impact of changes in operational conditions.

In Paper I we demonstrated, after careful system verification, that most methods applied to determine adsorption isotherms in LC could not be applied directly in SFC. This was mainly due to operational differences and to the fact that the fluid is compressible which means that everything considered constant in LC varies in SFC.

In Paper II we showed that the most accurate methods for adsorption isotherm determination in LC, the so-called plateau methods, do not work properly for SFC. Instead, methods based on overloaded profiles should be preferred.

In Paper III a Design of Experiments approach was successfully used to quantitatively describe the retention behavior of several solutes and the productivity of a two component separation system. This approach can be used to optimize SFC separations or to provide information about the separation system.

In Paper IV severe peak distortion effects, suspected to arise from injection solvent and mobile phase fluid mismatches, were carefully investigated using experiments and simulations. By this approach it was possible to examine the underlying reasons for the distortions, which is vital for method development.

Finally, in Paper V, the acquired knowledge from Paper I-IV was used to perform reliable scale-up in an industrial setting for the first time. This was done by carefully matching the conditions inside the analytical and preparative column with each other. The results could therefore provide the industry with key knowledge for further implementation of SFC.
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List of Papers

The thesis is based on the following papers, hereby referred to by their Roman numerals I-V


Reprints of Paper I-IV were made with permission from Elsevier.
My contribution to **Paper I-V** were as follows:

**I:** I did most of the planning, performed all experiments, made some of the calculations and wrote most of the article. **II:** I did most of the planning, performed most of the experiments and calculations and wrote most of the paper together with my co-authors. **III:** I did most of the planning, performed some experiments together with Dennis Åsberg, made parts of the calculations except DoE analysis and wrote most of the article together with Dennis Åsberg. **IV:** I did most of the planning, performed some experiments except those related to viscous fingering which were made by Jörgen Samuelsson and Andrew Shalliker. I made preliminary calculations and wrote most of the article together with my co-authors. **V:** I made most of the planning, experiments and calculations. I wrote most of the article together with my co-authors.
Manuscripts VI-XVII not included in the thesis:


**Abbreviations**

BINOL (1,1'-binaphthalene)-2,2'-diol  
BPR Back Pressure Regulator  
CFM Coriolis Mass Flow Meter  
DoE Design of Experiments  
ECP Elution by Characteristic Point  
FA Frontal Analysis  
HPLC High Pressure Liquid Chromatography  
IM Inverse Method  
LC Liquid chromatography  
MS Mass Spectrometry  
PDA Photo Diode Array  
PP Perturbation Peak  
RTM Retention Time Method  
SFC Supercritical Fluid Chromatography  
SMB Simulated Moving Bed  
TSO trans-1,2-diphenyloxirane  
TTBB 1,3,5-tri-tert-butyl-benzene  
UHPLC Ultra High Pressure Liquid Chromatography

**Symbols**

\( \alpha \) Selectivity  
\( C \) Solute concentration in mobile phase  
\( \rho \) Density  
\( F \) Phase ratio  
\( k \) Retention factor  
\( K \) Association equilibrium constant  
\( L \) Column length  
\( M \) Molecular weight  
\( \dot{m} \) Mass flow rate  
\( q \) Solute concentration in stationary phase  
\( q_s \) Monolayer saturation capacity (Langmuir)  
\( t_0 \) Void time  
\( t \) Time  
\( V_i \) Partial molar volume  
\( x \) Mole fraction, column position
1. Introduction

Chromatography is the unified name for techniques to separate groups of molecules or individual molecules, peptides or proteins from more or less complex mixtures. The purpose can be to identify and/or quantify and is then called analytical chromatography, or to purify and is then called preparative chromatography. In 2003 it was estimated that 5% of all chemical research involved chromatography [1]. The technique is generally classified as liquid chromatography (LC), gas chromatography (GC) or supercritical fluid chromatography (SFC), depending of the type of mobile phase. Chromatography is an incredible versatile technique which finds its applications in most fields, making it an indispensable tool in the realm of analytical chemistry. For example, the pharmaceutical industries rely on chromatography for quality control and assurance using analytical chromatography as well preparative chromatography for purification.

The outcome of the liquid or supercritical fluid chromatographic separation process is governed by the interactions between the solute and the mobile and stationary phase. In liquid chromatography, solvent mixtures of water, alcohols or organic solvents are used in combination with silica based stationary phases. Current trends in liquid chromatography entails the use of smaller particles, higher flows and pressures to reduce analysis times while maintaining efficiency [2]. SFC is another trend which offers decreased analysis time in analytical chromatography and increased productivity in preparative chromatography [3–6].
1.1 Supercritical Fluid Chromatography

1.1.1 General overview

Supercritical Fluid Chromatography is a chromatographic technique which utilizes a supercritical or subcritical fluid as main solvent. Historically, nitrous oxide, ammonia and carbon dioxide has been used, but also the noble gases argon and xenon as well as other hydrocarbons [6,7]. Chromatography using supercritical fluids was first described in 1962 by Klesper et al. [8]. In that work, the authors used mono- and dichlorodifluoromethane as mobile phase to separate porphyrins. Today, the typical implementation of SFC uses carbon dioxide. Carbon dioxide enters the supercritical defined state at and beyond 304.12 K (≈31 °C) and 74.5 bar.

There is a continuous ongoing debate about the name Supercritical Fluid Chromatography which may be misleading for most current typical applications where liquid co-solvents are added to the carbon dioxide which means that a supercritical phase is never reached and thus “Supercritical Fluid Chromatography” can be technically misleading [7,9,10]. Most major manufacturers of chromatographic instruments today provide equipment built and optimized for utilizing carbon dioxide [11]. There is little difference between HPLC or UHPLC and SFC instrumentations in terms of equipment, besides a modified pump to be able to pump chilled and compressed carbon dioxide as well as a back-pressure regulator (BPR) to maintain a particular density of the mobile phase [11].

The reason why SFC is an important chromatographic technique is related to the properties of the mobile phase [6,7]. Neat carbon dioxide or carbon dioxide with added co-solvent at sub- or supercritical conditions has lower viscosity ($\mu$), higher solute diffusion coefficients ($D_m$) and higher compressibility than comparable liquids used for liquid chromatography [6,7]. Some values of viscosities and adiabatic compressibility’s are summarized in Table 1. Literature suggests solute diffusion coefficients in neat carbon dioxide of a magnitude larger than in liquids [7].
Table 1: Comparison of solvent properties; data adapted and modified from [9] except for heptane [12]. Atmospheric pressure = 1.01 bar.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temperature [°C]</th>
<th>Pressure [bar]</th>
<th>Viscosity [cP]</th>
<th>Adiabatic compressibility $[10^{-5} \text{ bar}^{-1}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>20</td>
<td>138</td>
<td>0.09</td>
<td>37</td>
</tr>
<tr>
<td>CO₂</td>
<td>40</td>
<td>138</td>
<td>0.06</td>
<td>82</td>
</tr>
<tr>
<td>CO₂/MeOH 70/30 mol%</td>
<td>20</td>
<td>138</td>
<td>0.16</td>
<td>15</td>
</tr>
<tr>
<td>Water</td>
<td>20</td>
<td>1.01</td>
<td>1</td>
<td>4.6</td>
</tr>
<tr>
<td>Methanol</td>
<td>20</td>
<td>1.01</td>
<td>0.59</td>
<td>10</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>20</td>
<td>1.01</td>
<td>0.35</td>
<td>9.6</td>
</tr>
<tr>
<td>Heptane</td>
<td>20</td>
<td>1.01</td>
<td>0.41</td>
<td>11</td>
</tr>
</tbody>
</table>

The practical consequences of lower viscosity and higher solute diffusion coefficients are the possibility of operating at higher linear velocities than liquid chromatography or utilizing longer columns to obtain high efficiency. These consequences are beneficial for both analytical and preparative SFC. Higher compressibility means that properties such as density and temperature of the mobile phase can be altered by changing the pressure, which in turn will affect the chromatographic separation process. Furthermore, because there always exist a pressure drop along the column, there will be gradients of these properties along and across the column, something observed for both neat carbon dioxide or carbon dioxide with addition of methanol [13]. A simple schematic figure of the most important components in a SFC system are summarized in Figure 1 together with the typical gradients experienced in in SFC illustrated along the column.
Figure 1. Schematic figure of the major components in a SFC system. The occurrence and shape of typical gradients encountered in SFC are overlaid along the column, from inlet to outlet, left to right in the figure. Gradients of increasing volumetric flow, decreasing v% co-solvent, pressure, density and temperature are also illustrated (not to scale).

The most common detector in SFC is UV but also evaporative light scattering detectors, flame ionization detectors, polarimetric detectors and mass spectrometry [3,4,6,7] are used.

1.1.2 Application overview

SFC using packed columns has historically been utilized for a wide variety of applications. In general, SFC utilizes a less polar mobile phase, either neat CO$_2$ or CO$_2$ modified with a more polar co-solvent, classifying it as a normal phase separation technique [6]. The stationary phase is typically of porous silica type, for example bare silica, diol, amino-propyl, 2-ethylpyridine or chiral stationary phases [3,5–7], most of which are utilized in normal phase liquid chromatography.

Analytical SFC has been used for qualitative and quantitative chiral and achiral pharmaceutical analysis, analysis of pesticides, fossil fuels, polymers, peptides, natural products and more [4–7,14–16]. As noted by Lesellier and West [4] the historically documented applications of SFC may not accurately represent how analytical SFC is used today. Due to instrumental difficulties, for example detector noise due
to pumping and back-pressure regulation, SFC has had difficulties achieving sufficient accuracy and precision for GMP qualification [3]. With the advent of new and improved instrumentation as well as collaboration between instrument manufacturers and industry, SFC can and has been qualified for GMP operations [3,11]. However, detector noise in SFC systems is still worse than in LC systems [4].

The analytical applications of SFC are small in comparison to the dominating application; preparative chiral separations, see review articles [3,4,6,7,17]. Since the first reported chiral separation using SFC [18], it has grown into the dominating technique for obtaining enantiomerically pure material in the discovery phase in the pharmaceutical industry [3,4,17,19]. Today, the pharmaceutical industry routinely uses SFC for high-throughput screening and purification of chiral compounds [3,6,19]. Generally, g to kg amounts of compounds can be obtained using instrumentation that delivers flows between 10 g/min to 1 kg/min, using 1-10 cm inner diameter columns [3,6,17]. In almost all described applications of preparative chiral SFC it is used in batch mode and typically by stacking injections [17]. Simulated Moving Bed (SMB) applications in SFC has been reported and investigated by researchers but its complexity and cost of implementation has made its use limited [17]. Typically UV detection is used for detection and control of fraction collection but also MS.

The reason for the success of SFC in this field is because it can offer advantages over normal phase liquid chromatography. This is mainly due to the possibility of operating at higher linear velocities, i.e. shorter cycle time and hence increased productivity (purified amount per unit time). The cost benefit is not only related to decreased analysis time but also the reduced cost of mobile phase, i.e. predominantly carbon dioxide which can either be vented to the atmosphere or recycled [3,6]. Furthermore, purified components can be collected in a smaller volume, i.e. only the residual fraction of liquid co-solvent as the depressurization of the mobile phase allows carbon dioxide to be evaporated easily. This reduces the generation of liquid waste. Properties such as increased productivity and easier sample handling make the large scale use of preparative SFC interesting [3,6].
The number of reported publications of large scale SFC separations beyond the kilogram scale are limited especially because most such applications are confidential operations in the pharmaceutical industries [3,19]. As far as the author knows, the largest currently as of 2015 actively used preparative SFC unit is a system built by Novasep using a 20 cm inner diameter column by the company Johnson Matthey for a non-disclosed multi-component isomer separation problem [20]. Even larger scale systems of 35 cm inner diameter column has been reported but details remains unclear [3].

While the empirical evidence clearly has afforded SFC to become an important chromatographic tool, the fundamental knowledge of the technique is lacking. This was clearly laid out in the comprehensive review of SFC by the now late Georges Guiochon and Abhijit Tarafder [6] which was published around the same time the work on this thesis was started. Many researchers of SFC had also described a lack of interest in SFC by academy [4–6,11,21] with most applications taking place in the pharmaceutical industries.

1.2 Aim of study

From decades of research in LC it is well known that being able to quantify adsorption has been vital to the understanding and advancement of chromatography, hence Paper I-II were dedicated to the topic of adsorption isotherm determination methods. Particular focus was on the prerequisites for applying each method. While adsorption isotherms provide a physical chemical description of the separation process, a more rapid quantitative description in terms of its retention, productivity or arbitrary response, can be achieved by utilizing a Design of Experiments (DoE) approach. This was investigated in Paper III. The fundamental aspects of sample injection in SFC were investigated in Paper IV. Focus was put on quantitatively describing peak distortions observed in SFC. Utilizing the knowledge from Paper I-IV, the topic of scaling up SFC was investigated in Paper V where a chiral separation system was scaled up from a 4.6 mm inner diameter column to a 50 mm inner diameter column. The aim was to investigate what was needed to achieve a predictable scale-up in terms of maintaining the elution volume.
2. Theory and Methodology

2.1 Measurement of mass flow, pressure and temperature

Due to the compressibility of the mobile phase in SFC, the density and hence the volumetric flow rate can vary considerably along the flow path including the column in a SFC instrument [22,23]. If not measured, the volumetric flow can only be determined if the mass or molar composition of carbon dioxide and co-solvent and pressure and temperature are known. From these measurements the density of the mobile phase can be calculated and hence the volumetric flow rate. It must be emphasized that errors in either measurements or in the Equation of State (EoS) used to calculate density still poses an uncertainty in the calculation of the actual volumetric flow [22]. The basic knowledge of volumetric flow, pressure and temperature were deemed to be the most basic information needed in order to perform reproducible fundamental research in SFC. This is in clear comparison to LC where any reproducible study using commercially available instruments should be verified in terms of the accuracy of delivered flow rate and system void volumes [24,25]. A more particular reason would be because in order to utilize many dynamic methods of adsorption isotherm determination, the volumetric flow rate needs to be known and preferably constant. This can be verified by calculating the volumetric flow rate at the inlet and outlet of the column.

Today, no commercial SFC instrument except for some preparative instruments is equipped with mass flow regulation or read-out. Because of this it was decided to interface Coriolis mass flow meters (CFM) which presents a reliable way of measuring fluid mass flow [22,26]. The approach of measuring mass flow in using CFMs in SFC was reported in the 1980’s by Schoenmakers and Uunk and was revived in 2010’s by Tarafder et al. and later as well as by other authors [22,27–29].

On the typical analytical commercial SFC instrument, there are no more than a couple of pressure transducers, typically near the pump and at the back-pressure regulator. Rajendran el al. showed how the pressure drop was distributed in different sections of a custom built SFC system [30,31], clearly demonstrating why more pressure trans-
ducers would be required to quantify pressure drop over the column, but also the possibility of predicting the pressure drop at points not measured.

Temperature control on commercially available SFC instrumentations typically entails still or circulating air ovens with or without eluent heat exchangers which can be set at a constant temperature. Due to the adiabatic decompression of the mobile phase, the column can experience a significant temperature gradient with a lower temperature at the outlet of the column. This was demonstrated by Poe et al. [32,33] and later also by other authors [34,35]. Axial and radial temperature gradients were also measured and modeled by Kaczmarski and Poe [13]. While radial temperature gradients can lead to loss of efficiency due to an uneven flow profile across the column, axial gradients could change the local retention factor [36].

The SFC system used in Paper I-V was a Waters UPC² system (Waters Corporation, Milford, MA, USA), which can be considered as the third generation of commercially available SFC instruments [37,38]. The system was used in its basic configuration with a binary pump, a 2-column compartment, a Photodiode Array (PDA) detector and a back-pressure regulator. It maintains a constant low temperature at the pump which maintains carbon dioxide in its liquid state [7]. The system has two pressure transducers, one at the pump and one at the back-pressure regulator. For studies in Paper I-V except the methanol mass flow in Paper I, total and methanol mass flow was measured using one (Paper I-II) or two (Paper III-V) Coriolis flow meters. For all studies in Paper I-V the inlet and outlet pressure of column in use was measured using two absolute pressure transducers. Surface inlet and outlet temperature of the column in use was monitored with PT-100 resistance temperature detectors which were permanently attached to the column.
Figure 2. Schematic representation of how the external measurements of temperature, pressure and mass flow were made in the SFC system. In (a) it is shown how inlet and outlet pressure and temperature were measured. In (b) it is shown how mass flow $\dot{m}$ was measured outside the column using the Coriolis mass flow meter. Adapted from Paper I.
2.2 Calculation of density and methanol volume fraction

To determine the volumetric flow rate at a specified point in the flow path of an SFC instrument, measurements of mass flow, pressure and temperature must be complemented with calculations of density. In Paper I-V density was calculated using the Reference Fluid Thermodynamic and Transport Properties Database (REFPROP) v 9.1 program by the National Institute of Standards and Technologies [12]. REFPROP implements the EoS of Span and Wagner [39] and the mixture density of carbon dioxide and methanol using the mixing rules of Kunz et al. [40]. This approach has been reported by several authors [13,41]. For a 88/12 carbon dioxide methanol molar mixture, the error in density was estimated to less than 1.8 % at 40 °C and 150 bar [41].

In Paper I-V the average volumetric flow rate was used. This flow rate corresponds to the average of the flow rate at the inlet and outlet of the column. This approximation first assumes that the mass flow and fractions are constant, which could be verified by continuous measurements during experiments. It further requires that the pressure and temperature gradient along the column is kept at a minimum. This leads to the definition of near-isopycnic conditions, isopycnic meaning constant density:

\[
\rho(P_{inlet}, T_{inlet}) \approx \rho(P_{outlet}, T_{outlet}) \approx \rho(P_{average}, T_{average})
\]  

(1)

Where \( \rho \) is the density, \( P \) and \( T \) is the pressure and temperature at the inlet and outlet of the column. From the average density, the average volumetric flow rate can be calculated.
Another important parameter is the volumetric percent methanol. Because it is known to be the most important parameter controlling retention it SFC [7,9,42–45] it was imperative that it was verified properly. To calculate this we need to estimate the molar volume ($V$) of carbon dioxide and methanol [46]:

$$V = \frac{M}{\rho}$$

$$M = x_{CO_2} M_{CO_2} + x_{MeOH} M_{MeOH}$$

Where $M$ is the molecular weight of the fluid, $\rho$ is the density of the fluid and $x$ is the mole fraction. To estimate the volumetric fraction, the partial molar volume ($V_i$) needs to be calculated:

$$V_{CO_2} = V + x_{MeOH} \frac{\partial V}{\partial x_{CO_2}}$$

$$V_{MeOH} = V - x_{CO_2} \frac{\partial V}{\partial x_{CO_2}}$$

(3)

From the calculated molar volume and measured mass flows $\dot{m}$ of carbon dioxide and MeOH the volumetric fraction of MeOH can be calculated:

$$v^{\%}_{MeOH} = \frac{\frac{\dot{m}_{MeOH}}{M_{MeOH}} V_{MeOH}}{\frac{\dot{m}_{MeOH}}{M_{MeOH}} V_{MeOH} + \frac{\dot{m}_{CO_2}}{M_{CO_2}} V_{CO_2}} \cdot 100$$

(4)

The molar fractions were estimated using the measured methanol and total mass flow. The partial derivatives $\partial V/\partial x$ were numerically estimated [47].
2.3 Adsorption isotherms and their determination

One of the aims of the thesis was to investigate what methods of adsorption isotherm determination could be transferred from liquid chromatography. The adsorption isotherm refers to the quantitative description of a solute’s adsorption equilibria between the moving phase (mobile phase) and the stationary phase [48]. Adsorption isotherms are typically classified into different types. The type I adsorption models (Langmuir, Tóth, Jovanovicí) assumes homogenous or heterogeneous adsorption energy distributions. The Langmuir adsorption isotherm can be expressed as:

\[
q = q_s \frac{KC}{1 + KC}
\]

(5)

Where \( q \) is the concentration of the solute in the stationary phase, \( q_s \) and \( K \) is the monolayer saturation capacity and association equilibrium constant and \( C \) the concentration of solute in the mobile phase. The adsorption isotherm \( q \) is related to the retention time, \( t_R \) of a dilute injection:

\[
t_R = t_0 \left( 1 + F \frac{dq}{dC} \bigg|_{C=0} \right)
\]

(6)

Where \( t_0 \) is the void time and \( F \) the phase ratio which is the ratio of the stationary phase volume and the mobile phase volume. The adsorption isotherm is a key property for fundamental understanding, simulation and optimization of many chromatographic separation processes [48–56].

The retention time is related to the retention factor (\( k \)) and selectivity (\( \alpha \)) as follows:

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

\[
\alpha = \frac{k_2}{k_1}, k_2 \geq k_1
\]

(7)
An example of a bi-Langmuir adsorption isotherm (sum of two Langmuir terms) and the corresponding experimental and simulated elution profile is shown in Figure 3.

![Figure 3](image)

**Figure 3.** Figure illustrating the correlation between the elution profile (a) and the adsorption isotherm (b) of a 400 µL injection of 30 g/L omeprazole on an 250x4.6 10 µm amylose-based chiral stationary phase (CSP) in normal phase LC. In (a) the black line is the experimental profile and grey a simulated profile while in (b) the black line represents the adsorption isotherm of the first eluting enantiomer of omeprazole while the grey represents the later eluting. Modified from Paper VII.

Methods to determine adsorption isotherms in liquid chromatography are well characterized and understood [48,57]. One method is frontal analysis (FA) which is considered a reference method [48,57]. The FA method uses the breakthrough volumes of a number of plateau reaching injections of increasing concentrations to determine points on the adsorption isotherm. Another is the perturbation peak method (PP) which has been shown to be as accurate or even more so as FA [48,58–62].

The perturbation peak method exploits the theoretical relation between the retention time \( t_{R,i} \) of a small injected deficiency or excess concentration pulse on a concentration plateau \( C_i \) and the slope of the adsorption isotherm for this plateau concentration \( dq_i/dC_i \).

\[
t_{R,i} = t_0 \left( 1 + F \frac{dq_i}{dC_i} \right)
\]  

(8)
By establishing a number of plateaus and measuring the retention times the adsorption isotherm \( q \) can be determined by regression. The PP method can be used to determine single or multicomponent competitive adsorption isotherms.

![Figure 4](image-url)  

**Figure 4.** Illustration of the principles of the perturbation peak method (PP). The top subplot shows the retention times of different perturbation peaks, beginning at the zero plateau \( t_{R,1} \), where the column is equilibrated with pure mobile phase. The bottom subplot shows the relation between the slope of adsorption isotherm \( dq_i/dC_i \) and the retention time of the perturbation peak \( t_{R,i} \) on a plateau concentration \( C_i \).
Other methods frequently utilized are the Elution by Characteristic Points (ECP) Method [48, 63, 64], Retention Time Method [48, 65–67], Inverse Method (IM) [54, 68, 69]. The ECP, RTM and IM all extracts adsorption data from overloaded elution profiles. The ECP uses the retention times of concentration zones of the diffuse part of elution profiles which correlates to the slope of the adsorption isotherm for each concentration.

The RTM uses the retention factor and the retention time of the fronts of elution profiles for injections of different volume and/or concentration. The difference between experimentally obtained front retention times and calculated times using an adsorption isotherm model is then minimized until valid adsorption isotherm parameters are found.

Both the ECP and RTM methods are derived from the ideal model of chromatography [48] why the error in their application will increase with decreasing column efficiency. The IM is based on iteratively solving a column mass balance model, for example Equation 9, where the elution profile of the solute(s) is described by an adsorption isotherm. The parameters of the adsorption isotherm are changed such that eventually the simulated and experimental chromatograms overlap. The adsorption isotherm parameters for which this occurs is then said to describe the separation system.

The number of publications concerning the determination of adsorption isotherms in SFC is still small but growing [70–75]. All adsorption isotherm determination methods require precise control and knowledge of volumetric flow, temperature, system and column void volumes [24, 25, 76].
2.4 Simulation of chromatographic processes

When the adsorption isotherm of a solute is known, it is possible to model the separation process in terms of convection, dispersion and adsorption and simulate the propagation of a solute through a column [48]. One model that is often applied to small molecule separation systems with sufficiently high column efficiency is the so called Equilibrium-Dispersive (ED) model [6] often referred to as “the simplest realistic model of chromatography” [6]. The choice of column model must however be made not to oversimplify, or misunderstandings of the process can be made [77]. The ED model can be formulated as follows.

\[
\frac{\partial C_i(x,t)}{\partial t} + F \frac{\partial q_i(x,t)}{\partial t} + u \frac{\partial C_i(x,t)}{\partial x} = D_{a,i} \frac{\partial^2 C_i(x,t)}{\partial x^2}
\]

(9)

where \(C_i(x,t)\) and \(q_i(x,t)\) are the concentrations of substance \(i\) at time \(t\) and position \(x\) in the mobile and stationary phase, \(F\) is the phase ratio, \(u\) is the mobile phase linear velocity and \(D_{a,i} = \frac{L \cdot u}{N_i}\) is the lumped mass transfer and dispersion coefficient, \(N_i\) is the column efficiency. \(\Phi_i\) is the injection profile. The model can be solved numerically by many different approaches [68,78,79] and [VI]. The number of publications in SFC using simulations in an effort to predict experimental data limited [72,73,80] and [I-IV].
2.5 Design of Experiments

The concept of Design of Experiments, DoE, refers to a methodology to design a minimal number of experiments to obtain a maximum amount of information about the studied system [81]. **Paper III and V** utilized a full factorial design with three factors; methanol level, pressure and temperature. The response which was studied was the retention factor \((k)\), selectivity \((\alpha)\) and productivity (purified amount per unit time). From literature it is known that the retention factor has a quadratic relationship with the methanol content \([42–45]\). It is also known that there are interactions between factors such as pressure and temperature in how they affect retention. Based on this a two level design would be insufficient [81]. A three level design which varied the factors at a low, middle and high was selected. This means at least \(3^3\) runs plus additional center point runs.

The regression model used to fit the responses is on the following form:

\[
Y = p_0 + p_1 C + p_2 P + p_3 T + p_4 CP + p_5 CT + p_6 PT + p_7 C^2 + p_8 P^2 + p_9 T^2 \tag{8}
\]

Where \(Y\) is the response (retention factor, selectivity or productivity), \(p_0-p_9\) constants, \(C\) the methanol concentration (v%), \(P\) pressure and \(T\) temperature. Coefficients were estimated using multiple linear regression and the regression models were evaluated using analysis of variance (ANOVA). All calculations were performed using MODDE version 7 (Umetrics, Umeå, Sweden).
3. Discussion of papers

3.1 Word analysis of Paper I-V

By analyzing the occurrence of words in Paper I-V, the superficial reader can understand that the focus of this thesis has been on adsorption and how to determine the isotherms. Methanol has exclusively been used as co-solvent. Temperature and pressure has been investigated.

![Word cloud](https://github.com/amueller/word_cloud)

**Figure 5.** Word analysis of Paper I-V presented as a word cloud where the size of the font is correlated to the frequency of occurrence of certain words. Bigger font means more frequent occurrence. Data generated using Word Cloud Python ([https://github.com/amueller/word_cloud](https://github.com/amueller/word_cloud), accessed September 2015)
3.2 Paper I

The motivation of this paper was the lack of knowledge about how to characterize adsorption in SFC. The number of previous works \([70-73]\) was limited and in general lacked a thorough discussion about the applicability of the methods used. Three main reasons were suspected to be (i) the lack of fundamental SFC understanding, (ii) the lack of commercial SFC systems properly evaluated for fundamental studies and (iii) the previous relatively low interest in the SFC area. A few notable studies could be found. Depta et al., Lübbert et al., Ottiger et al., Wenda et al. and Bao et al. used the FA, PP, IM, IM and ECP method respectively to determine adsorption isotherms of different solutes in mixed carbon dioxide/co-solvent mobile phases \([70-73,80]\). Some like Ottiger et al. and Wenda et al. had a discussion about density gradients; others like Lübbert et al. approximated mixed phase densities with that of neat carbon dioxide.

In the planning of the study it was decided to evaluate four different methods, the Perturbation Peak method (PP) \([48,58,61,82,83]\), the Elution by Characteristic Points (ECP) \([48,63,64]\), the Inverse Method (IM) \([54,68,69]\) and the Retention Time Method (RTM) \([48,65,66,84]\). As model system, the retention of 1,2-Dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (antipyrine) on a bare silica column was studied. The set experimental conditions were 90/10 v% CO\(_2\)/methanol at 150 bar and 35 °C. The set volumetric flow was 1 mL/min.

Particular focus was paid to the prerequisites for applying each method. The basic criterion of any experiment must of course be to know the actual conditions. As was explained in Section 2.1, pressure, temperature and mass fraction affects both density and volumetric composition of the eluent. Studies have demonstrated the importance of verifying and estimating the pressure drop \([30]\), the variations of mass and volumetric flow of commercial systems \([28]\) and combined pressure, temperature and density drop in SFC \([13]\).
Table 2. Temperature (T), pressure (P), average volumetric flow (u_{total}) and density (\rho) at the column inlet and outlet together with mass flow of CO_2 (m_{CO_2}) and of MeOH (m_{MeOH}). Reproduced and reprinted with permission of Elsevier from Paper I.

<table>
<thead>
<tr>
<th></th>
<th>T [°C]</th>
<th>P [bar]</th>
<th>u_{total} [mL/min.]</th>
<th>\rho [g/mL]</th>
<th>m_{CO_2} [g/min.]</th>
<th>m_{MeOH} [g/min.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column inlet</td>
<td>34.5</td>
<td>161</td>
<td>1.07</td>
<td>0.860</td>
<td>0.81</td>
<td>0.11</td>
</tr>
<tr>
<td>Column outlet</td>
<td>34.2</td>
<td>156</td>
<td>1.07</td>
<td>0.858</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>34.4</td>
<td>158.5</td>
<td>1.07</td>
<td>0.859</td>
<td>0.81</td>
<td>0.11</td>
</tr>
</tbody>
</table>

The Waters UPC^2 system was modified by interfacing pressure transducers and temperature probes on the column inlet and outlet. Carbon dioxide mass flow was measured using a Coriolis mass flow meter. Methanol mass flow was measured by weighing the amount pumped over time.

In Table 2 the measured and calculated conditions are presented. Based on this data it was concluded that experiments conducted could be considered near isothermal, isobaric and isopycnic. The consequence of this is that retention will not vary along the column and both the PP, RTM, ECP and IM can be applied directly. Perturbation peak experiments were performed by pumping 10 stock solutions between 10 and 150 g/L in the 10 v% co-solvent line and perturbing the system with stock solution diluted 10 times. Perturbations on zero to 19.2 g/L plateaus are presented in Figure 6.
Figure 6. Perturbation peak results: in (a) an overlay of four 3 µL injections of 1 g/L antipyrine in neat MeOH in a stream of CO2/MeOH, in (b) an overlay of four 3 µL injections of 1 g/L antipyrine on a plateau of 1.3 g/L, in (c) an overlay of four 3 µL injections of 5 g/L antipyrine on a plateau of 6.4 g/L and in (d) an overlay of four 3 µL injections of 13 g/L antipyrine on a plateau of 19.2 g/L. Reproduced and reprinted with permission of Elsevier from Paper I.

ECP experiments were performed by injecting 50 µL of 250 g/L antipyrine while RTM and IM experiments by injecting different volumes of varying concentration.

The corresponding bi-Langmuir adsorption isotherms are presented in Figure 7 where it is apparent that the data obtained by ECP, RTM and IM are very similar and that PP data is different. Using the obtained data to simulate overloaded elution profiles shows that either ECP, RTM or IM can be used to predict this data if using the Equilibrium-Dispersive model of chromatography. Different reasons to why the data obtained using the PP data could not be used to predict elution profiles within the investigated concentration range were discussed. One likely explanation was that the methanol volume fraction
differs at each plateau. The conclusion of the study was therefore that the PP method is not a suitable method for SFC. Instead, methods using the elution profiles should be preferred.

**Figure 7.** Adsorption isotherms obtained by the Perturbation Peak method (PP, solid black), Elution by Characteristic Point method (ECP, dashed black), Retention Time Method (RTM, dash-dotted black) and the Inverse Method (IM, solid gray). The inset shows the Perturbation Peak raw slope data (symbols) and the best fit of the Bi-Langmuir adsorption isotherm (black line). Reproduced and reprinted with permission of Elsevier from **Paper I**.
3.3 Paper II

As a consequence of the observations in Paper I about the Perturbation Peak method (PP) it was decided to perform an in depth study of why adsorption data obtained using the PP method could not describe elution profiles as well as data obtained using ECP, RTM or the IM. The main hypothesis was that since the instrument pumps co-solvent at a constant rate, the actual methanol content will decrease as concentration of antipyrine increases. Hence, each perturbation peak corresponds to a point on the adsorption isotherm for a particular methanol volume fraction. The actual volume fraction methanol can be calculated according to Equation 9 where \( m_{\text{tot}} \) is the weight of a volume \( V_{\text{tot}} \) containing \( m_{\text{antipyrene}} \) dissolved in methanol with density \( \rho_{\text{MeOH}} \). Solutions of concentrations of up to 100 g/L antipyrine were prepared and their methanol volume fraction calculated, see Table 3.

\[
v\% = \frac{m_{\text{tot}} - m_{\text{antipyrene}}}{\rho_{\text{MeOH}} V_{\text{tot}}} \cdot 100
\]

The prepared concentrations were chosen in such a way that the deviation could be compensated by the pump which has a minimum change of 0.1 v%. For example, if 10 v% methanol plateau is sought and the concentration of antipyrine in the co-solvent is about 60 g/L the volume fraction methanol is about 95 v% and the system needs to pump 10.5 v% to maintain 10 v% methanol volume fraction.

<table>
<thead>
<tr>
<th>Antipyrine [g/L]</th>
<th>0</th>
<th>13.52</th>
<th>25.59</th>
<th>37.43</th>
<th>49.05</th>
<th>60.46</th>
<th>71.67</th>
<th>82.67</th>
<th>93.47</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH volume fraction [-]</td>
<td>1</td>
<td>0.990</td>
<td>0.981</td>
<td>0.971</td>
<td>0.963</td>
<td>0.954</td>
<td>0.945</td>
<td>0.937</td>
<td>0.928</td>
</tr>
</tbody>
</table>

When plotting the retention times of each perturbation peak in theory they should overlap on the rear slope of an overloaded elution profile at the same concentration. In Figure 8 it is apparent that the pertur-
bation peaks obtained when pumping the same percentage co-solvent irrespectively of the antipyrine concentration does not overlap but instead predicts longer retention of the equivalent concentration. The deviation does however increase with increasing antipyrine concentration on each plateau. When maintaining the same methanol volume fraction on each plateau by compensating by changing the pump flow, the correspondence is better but still not exactly following the diffuse rear. The conclusion was therefore that the initial hypothesis could not be confirmed. What was clarified using the ECP method was the strong dependence of antipyrine retention to the volume fraction methanol, see Figure 9.

**Figure 8.** In (a) experimental elution profiles for injections of 250 g/L antipyrine at different volumes using an instrumental setting of 10 v% MeOH. The symbols are the perturbation peak retention times obtained from perturbation injections on established antipyrine plateaus. The circles using the “standard” approach with set modifier fraction of 10 v% MeOH and the squares using the “compensated” approach. In (b) ECP adsorption isotherm slopes (solid line), symbols are experimental data from perturbation peaks at an instrumental setting of 10 v% MeOH (circles) or using compensated MeOH fractions (squares) with fitted bi-Langmuir model (dashed and dotted line). In (c) the adsorption isotherms obtained using the ECP (solid grey), PP uncorrected (dotted), PP corrected (dashed) and FA (circles) methods. Reproduced and reprinted with permission of Elsevier from Paper II.
Figure 9. In (a) the natural logarithm of the retention factor for antipyrine (k) is plotted for 8, 8.5, 9.1, 9.6, 10, 10.5, 11, 11.5 and 12 v% MeOH in the eluent (circles) and the line is the best fit of the data to Eq. (8). In (b) the adsorption energy distributions calculated for adsorption data determined using the ECP method for the same MeOH compositions as in figure (a). In (c) the bi-Langmuir model fit to the ECP adsorption data at the same eluent compositions as for figure (a). Reproduced and reprinted with permission of Elsevier from Paper II.
3.4 Paper III

Understanding retention in SFC is vital to the design and optimization of separation systems and to evaluate method to characterize adsorption and retention and adsorption was therefore the purpose of Paper I-II. The purpose of Paper III was to propose and evaluate a methodology to study the convoluted effects of pressure [7,85], temperature [6,43,86,87] and methanol content [7,9,42–45] on the separation of two small neutral racemic compounds on a chiral column (Kromasil Cellucoat). This is important aspect besides studying how the stationary phase effects retention in SFC [86,88–93]. An exploratory Design of Experiments (DoE) approach was used to study the main factors and their possible interactions. Based on the observations from Paper I-II, particular attention was directed to account for the discrepancies between system set and actual values inside the column as well as maintaining close to isobaric, isothermal and isopycnic conditions. To calculate the retention factor and selectivity, the void volume (void time multiplied by the volumetric flow) of the column [94] must be determined.

![Figure 10](image)

**Figure 10.** The void volume determinations using pycnometry (dashed line), and injections of nitrous oxide, 1,3,5-tri-tert-butyl-benzene (TTBB) and MeOH (bars), are presented for 2.5, 5.0 and 7.5 v% MeOH. The volumetric flow rate was set to 0.7 mL/min but the elution volume was calculated from the actual estimated volumetric flow rate for each experiment from the measured mass flow and density of the mobile phase. The back pressure was set to 160 bar and the temperature was set to 30°C. Reproduced and reprinted with permission of Elsevier from Paper III.
Based on a limited number of investigations dedicated to this, three dynamic and one static method were evaluated [95,96]. In Figure 10 it is apparent that the retention volume of nitrous oxide [95] is constant between 2.5 to 7.5 v% methanol while the same volume for both methanol and 1,3,5-tri-tert-butyl-benzene (TTBB) [97,98] varies. Based on this observation, the void volume derived from the elution time of nitrous oxide was used in calculating the retention factor.

A three-level full factorial design was used in order to accurately model the response within the boundaries of the design, also with the anticipation of both quadratic and interaction terms. The design was spanned by 120, 160 and 200 bar, 24, 30 and 36°C, and 2.5, 5 and 7.5 v% methanol for trans-1,2-diphenyloxirane, (TSO) or 15, 20 and 25 v% methanol for (1,1'-binaphthalene)-2,2'-diol (BINOL). Note that these values correspond to instrument set values. Retention factors were calculated for each point in the design region. Analysis of data showed that methanol was the most important factor controlling retention of both enantiomers of BINOL and TSO as is seen by the negative coefficient bars in Figure 11. Additionally, a quadratic methanol term was found to be the third most important factor. The second most important factor for both enantiomers of TSO and BINOL was pressure, also indicated by negative bars in Figure 11. The selectivity for TSO was found to be most affected by methanol content, while for BINOL temperature. Both pressure and temperature had opposite effect on the selectivity for BINOL and TSO.

At each point in the design space, injections of 12-22 µL of 40 g/L TSO were made. The maximum injection volume fulfilling 100% yield and purity was used to calculate the productivity [48,99] for the collection of either enantiomer in so called stacked injection mode. Analysis of variance revealed that the most important factor was the methanol content followed by temperature, for both an increase gave increased productivity, see Figure. 12.
Figure 11. Centered and normalized coefficients from the model fit for the first and second retention factor, respectively, for: a) TSO and b) BINOL. The error bars represent the 95% confidence interval of the coefficients. Reproduced and reprinted with permission of Elsevier from Paper III.

Figure 12. a) Centered and normalized coefficients with 95% confidence interval from the model fit to the productivity for the optimum touching-band chiral separation of TSO is plotted. In b) the productivity is plotted as a function of amount of modifier in the eluent and the temperature. Reproduced and reprinted with permission of Elsevier from Paper III.

In conclusion, the study presented important insights into how to properly study analysis of variance for important factors in SFC. The exploratory results obtained can be used for method optimization or as foundation to fundamental studies investigating the underlying mechanisms.
3.5 Paper IV

All column based chromatography is based on the application or injection of sample into the mobile phase stream. To minimize distortions of elution peaks the injection solvent in LC should be identical to the eluent or peak deformations can follow [100–102]. This includes properties such as solvent composition and pH. In SFC, the current practice is to inject in mixed stream. This means that different diluents will always be injected, either with dissimilar or similar elution strength as the mobile phase. The purpose of the paper was to investigate and quantify the origins of peak deformations caused by the injection, a well-known phenomena albeit investigated by few authors in SFC [103–105].

In SFC there are at least two principally different injection methods [17,106]. One is the mixed stream and the other the modifier stream injection, see Figure 13. For both methods, a sample loop is filled with sample and eventually transferred onto the column. Mixed stream injection is similar or identical to how sample is injected in liquid chromatography, the total flow of carbon dioxide and co-solvent transports the sample plug onto the column, see Figure 13(a). For modifier stream, it is the stream of co-solvent which transfers the sample from the loop, to the mixing vessel and finally onto the column.

Figure 13. Schematic figure illustrating instrumental plumbing for (a) mixed stream injection and (b) modifier stream injection. In all experiments the mixer corresponds to a 250 µL passive mixer in the Waters UPC² system. Reproduced and reprinted with permission of Elsevier from Paper IV.
Initial experiments were performed to qualitatively investigate the shape of the elution profiles of injections performed in mixed and modifier stream injections. For this purpose, two neutral probes were studied. 0.5, 30 and 75 µL of 0.2 and 100 g/L antipyrine and 0.5 and 20 g/L 2-Hydroxy-N-phenylbenzamide (salicylanilide) were injected separately in mixed and modifier stream injections. Studying antipyrine in Figure 14(a) it is apparent that severe peak distortion takes place for injection volumes over 5 µL. Only when injecting 5 µL in mixed and modifier stream mode is the elution profile identical. It is apparent that elution profiles obtained in mixed stream mode shifts the center of mass to shorter retention. The same phenomena are observed for injections of more concentrated solutions, but more sharpening of the front is observed.

![Figure 14. Comparisons between mixed (solid black line) and modifier stream (solid grey line) injections of antipyrine and salicylanilide. In the top row 5, 30 and 75 µL injections of antipyrine. In (a) 0.25 g/L and in (b) 100 g/L. In the bottom row 5, 30 and 75 µL injections of salicylanilide. In (c) 0.5 g/L and in (b) 100 g/L. Reproduced and reprinted with permission of Elsevier from Paper IV.](image-url)
To investigate the correlation of distortion with the diluent, 0.2 g/L antipyrine was prepared in methanol, ethanol and toluene. 75 µL injections of the sample were made and it could be noted that using methanol as diluent gave the most severe peak distortion, followed by ethanol and toluene, see Figure 15(a). A similar experiment was performed in NPLC, where the same column was used but antipyrine was eluted using 15/85 v% ethanol/heptane. 0.2 g/L antipyrine was prepared in the eluent, ethanol and isopropanol. Injections showed that the least peak distortion was observed using the eluent, followed by isopropanol and ethanol. The important conclusion from these experiments are that the peak-distortion phenomena likely are similar in SFC and NPLC and related to the eluent strength of the injection solvent compared to that of the mobile phase.

Figure 15. Observations of the peak distortion of antipyrine and salicylanilide when injected in SFC and NPLC mode. In (a) experiments conducted in SFC mode with 75 µL ca 0.2 g/L antipyrine injected in toluene (grey), ethanol (dashed grey) and methanol (black). Running conditions were 90/10 v% CO۲/MeOH. In (b) experiments conducted in NPLC mode with 75 µL of ca 0.2 g/L antipyrine injected in 2-propanol (dashed-grey), ethanol (black) and 85/15 v% heptane/EtOH (gray). Running conditions 85/15 v% heptane/EtOH. In (c) and (d) the equivalent injections of 0.2 g/L salicylanilide. Reproduced and reprinted with permission of Elsevier from Paper IV.
If the elution strength that is the most important factor contributing to the peak distortion, the distortion should be able to be simulated using the Equilibrium-Dispersive model of chromatography. This also requires that the adsorption isotherm has a quantifiable dependency on the methanol content in the eluent. This dependency was investigated by injecting 5 μL of 300 g/L antipyrine at different methanol levels from 7.2 to 100 % and then extracting the adsorption data by using the ECP method. While the monolayer saturation capacity decreases with increasing methanol content, the association equilibrium constant has a more complex relationship. To quantitatively describe the dependency, a cubic polynomial was used to describe the relationship of both the monolayer capacity and the association equilibrium constant.

Using the determined adsorption isotherm and its dependency of the methanol content as well as the broadening of the injected methanol plug, it was possible to quantify the observed peak distortion for different injection volumes at low concentration of analytes, see Figure 16. Simulations did not predict the experimental outcome exactly which was attributed to the possibility of occurrence of viscous fingering [107–110], a phenomena well known from liquid chromatography. Liquid chromatography experiments mimicking the viscosity ratio observed in SFC was undertaken, strengthening this hypothesis.
Figure 16. Experimental (a) and simulated (b, c) elution profiles of antipyrine is plotted. In (a) experimental elution profiles for 2, 5, 10, 20, 30, 60 and 75 \( \mu \text{L} \) 0.25 g/L antipyrine in eluent containing 7.2 v\% MeOH are plotted. In (b) the corresponding simulated injections when the methanol plug is not retained and (c) same as in (b) but now the methanol is retained. Reproduced and reprinted with permission of Elsevier from Paper IV.
3.6 Paper V

One of the most important applications of SFC today is preparative chiral chromatography where g to kg amounts of compounds needs to be purified [3,6,17,19]. Liquid chromatography has been the *de facto* standard to obtain enantiomerically pure material in early pharmaceutical development [99,111]. Chromatography is a very scalable technology which has been proven to reliably scale up a million times [99]. This merit of chromatography means that method development and optimization can be carried out in small laboratory scale where the consumption of materials and energy is minimized and subsequently scaled up to a scale which satisfies the needed throughput.

Although preparative SFC is such an important application, it remains relatively little investigated, in particular concerning the topic of scaling up [112]. Because of the compressibility of the mobile phase and the known correlation between retention shifts and the gradients caused by this compressibility any difference in column length or particle size between the small and large scale system will hinder reliable scale-up. Tarafder et al. has suggested the concept of average density matching to compensate for such differences [112]. Furthermore, as there are different manufacturers of small and large scale systems, their design in terms of flow regulation, mixing of carbon dioxide and co-solvent and system pressure contribution, the situation becomes complicated.

The topic of Paper V was to investigate if a reliable i.e. preserved elution volume scale-up could be made from one small scale system (Waters UPC²) to one preparative system (Novasep SuperSep 600) by using a 250x4.6 mm column scaled to a 250x50 mm packed with the same chiral stationary phase. An overview of how this approach was made is presented in Figure 17.
A separation system consisting of the chiral resolution of trans-1,2-diphenylethylene oxide (TSO) was chosen as model system. The chiral stationary phase was chosen based on what column was available at AstraZeneca R&D Mölndal [19] and at the same time could be packed in a smaller diameter column using the same batch. The first step was to investigate which parameter had the greatest impact on the retention factors and selectivity of the enantiomers of TSO, using the DoE approach of Paper III. The methanol level was found to be the most...
important parameter for both retention factors, followed by pressure and temperature. Selectivity was most affected by temperature, followed by methanol level and pressure. The conclusion was that a scale-up must preserve all parameters, but most importantly methanol content. Because the retention factor only corresponds to the initial slope of the adsorption isotherm, it was decided to verify the conclusion using high concentration and high volume injections on the 50 mm inner diameter column. The experiments could well confirm the DoE conclusion, see Figure 18.

**Figure 18.** Figure showing large scale elution profiles at a 20 % positive or negative change of methanol fraction, pressure and temperature after the injection of 4 mL of 40 g/L TSO. The solid gray line is the reference chromatogram acquired at 8.5 wt.% methanol, a BPR of 132 bar and temperature at 32 °C. In (a) the methanol content is increased 10.2 wt.% (dotted line) or decreased to 6.8 wt.% (dashed line). In (b) the BPR pressure is increased to 158 bar (dotted line) or decreased to 106 bar (dashed line). In (c) the temperature is changed to 38.4 °C (dotted line) or to 25.6 °C (dashed line). The preparative SuperSep 600 instrument system was used. Reproduced and reprinted from Paper V.
The core of the approach of scaling-up was to transfer the exact conditions in terms of flow, methanol content, pressure and temperature between the 4.6 and 50 mm column. Because the SuperSep 600 is controlled using built in Coriolis mass flow meters, which also acts as an automatic control system of the pumps, the mass flow on the UPC² system must be determined. This was done as described in Paper I-IV. The determined mass flow and methanol mass fraction on the UPC² system could then directly be scaled by a factor of 118 to the SuperSep 600 system. This is the column volume increase going from inner diameter 4.6 mm to 50 mm. To match the pressure drop along the column in both systems, inlet and outlet pressures were monitored and the back-pressure on both systems adjusted to a value for which the pressure gradient matched. The same approach was made using the surface temperature measurements.

Differences in system void volume were accounted for by matching the retention time of TTBB on both systems. Having matched the column length, packing material, mass flow and fraction methanol, pressure and temperature gradient on both systems, injections of identical load were made. Their analytical retention volumes were found to match well, see Figure 19. However, distinct fronting of the overloaded elution profiles obtained on the 50 mm column was observed. At present, no confirmed explanation to this observation exists. Nevertheless, the conclusion from Paper V demonstrated that scale-up in SFC can be made more reliable, albeit not completely predictable.
Figure 19. The results from the matched scale-up at different eluent methanol level in wt.% units: (a) 4.2 (b) 8.5 and (c) 12.8. The black lines in all figures/insets correspond to injections on the analytical (UPC²) unit with the 250 x 4.6 mm column and the gray lines correspond to injections on preparative (SuperSep) unit with the 250 x 50 mm column. The insets correspond to analytical injections using the analytical UPC² and respective the preparative SuperSep unit, respectively. The main figure corresponds to different injected volumes of 40 g/L TSO on the analytical UPC² and on the preparative SuperSep unit. Corrections were made for system void volumes by matching the elution time of the TTBB marker. Reproduced and reprinted from Paper V.

To illustrate the complexity of SFC separations, a seemingly simple experiment was devised on the analytical system. Elution profiles for equal injections of TSO were recorded at 1 and 4 mL/min. In order to match the elution volumes of the chromatograms obtained at the different flows, both the column average pressure and the methanol volume fraction delivered at each flow had to be adjusted using both calculations of density and methanol content.
4. Concluding remarks

The expression “two steps forward one step back” rather precisely summarizes the work undertaken in this thesis. Everything began with the basic question if reproducible research could be performed using the latest generation commercial SFC instrumentations. Based on previous work by several authors, some of whom built their own SFC systems, the answer was clearly no. The compressibility of the mobile phase made user set parameters such as volumetric flow, pressure and even modifier content ambiguous. At which point in the flow path were these parameters valid, at the pump, at the inlet of the column or at the back pressure regulator?

The approach of interfacing Coriolis mass flow meters was the first attempt of making the research more reproducible because mass flow can be reported unambiguously. By coupling pressure transducers and temperature probes at the inlet and outlet of the column, experimental conditions can be even more unambiguously reported. Together, these measurements also allow for calculation of the fluid density, which in turn can be used to calculate the volumetric flow rate. Because the calculation of density relies on the accuracy of the Equation of State, this parameter must still be used with caution, as its error is only known for certain conditions. An investigation of the accuracy of the available Equation of States with mixing rules should be investigated further. Additionally, density can only be calculated for a limited amount of mixtures, why more basic research is needed.

Nevertheless, the additional measurements allowed us to find so called near-isopycnic (also near isothermal) where methods to determine adsorption isotherms typically used in LC could be directly transferred to SFC. The results were interesting but at the same time discouraging, as it revealed that the most accurate methods in LC are very complicated or maybe impossible to use in SFC. To use the perturbation peak methods, solute dissolved in the liquid modifier will displace the liquid and if no adjustment is made, each increased concentration plateau will decrease modifier level. The adsorption data is likely valid but each obtained point represents a unique point on an adsorption isotherm for a particular modifier content. This data can-
not be used to simulate overloaded injections in isocratic elution mode. Even if it was shown that adsorption isotherm determination methods which rely on the use of overloaded elution profiles could be used to predict elution profiles of varying injection volume, Paper IV made it clear that the possibility of elution profile distortion must always be taken into account. Furthermore, Paper I-II also relies on the assumption that co-solvent adsorption is implicitly accounted for in the adsorption isotherm. This may or may not be valid over different ranges of co-solvent levels and/or stationary phases and remains to be investigated. Further research into methods to determine adsorption isotherms in SFC is necessary to gain deeper insight into the separation process, as has been shown numerous times in LC.

Due to the findings of Paper I-II, Paper III was devoted to a more descriptive approach, i.e. the use of Design of Experiments to quantify the performance of the separation process. The attempt was successful and could be used to model the response within the design space. This is an important observation since it would allow experimenters to use the predictive power within the design space to for example optimize the conditions for a preparative separation as was demonstrated in the paper. As one can fit any response given a sufficient number of parameters, caution must of course be taken when extrapolating beyond the design space.

The observant reader may have noticed that all experiments in Paper I-IV were performed at low flow rates, which may seem odd given that one of the best aspects of SFC is its possibility to operate at very high flow rates. The reason for this is that methods of adsorption isotherm determination otherwise would need to take into account gradients in density, flow rate, temperature. This would be very challenging but the recent work by Kaczmarski et al. shows that it not impossible.

Paper V utilized SFC at higher volumetric flows and managed to present a viable approach for scaling-up separation systems in SFC. By scaling up SFC using the same length of column, no calculation of mobile phase density is required if mass composition and pressure drop are preserved during the scale-up.
To summarize I would like to quote Stellan Hjertén “No doubt the final success is, in most cases a consequence of a series of consecutive advances and improvements and, therefore, not concentrated in one or a few days”

SFC is a usable technique that has found, and will continue to find suitable applications where it in some aspects will outperform liquid chromatography. Its ultimate success will require a continued research effort, which today actually seems promising.
6. Swedish Summary

Kromatografi är en kemisk separationsteknik som baserar sig på att de ämnen, eller molekyler, som skall separeras ifrån varandra fördelar sig på olika sätt mellan en rörlig (mobil) fas och en stillastående (stationär) fas. Vid vätskekromatografi är den rörliga fasen en vätska, ofta vattenbaserade lösningar innehållande salter eller organiska lösningsmedel med definierat pH. Den stationära fasen består ofta av små porösa kisel-dioxidpartiklar (små pärlor) som modifierats kemiskt på olika sätt. Den stationära fasen nedpackas i en stålcyliner med en diameter på cirka 3-5 mm och en längd på 50 – 150 mm. Den rörliga fasen pumpas vid mer eller mindre högt tryck alltifrån cirka 50 bar till nästan 1000 bar.


Vid preparativ kromatografi är syftet renframställning istället för kvantitativ information, och stora mängder substans introduceras vid provinjektionen. De höga koncentrationerna medför att substansernas kromatografiska toppar blir överladdade; den högre koncentrationen som finns i toppen strävar efter att komma ut snabbare vilket medför att toppens framsida blir skarp medan baksidan tar formen av en skidbacke. Fenomenet kallas ”svansning” och beror på att antalet adsorptionsställen – ställen som molekylerna kan fastna på – är begränsade hos den stationära fasen i kolonnen. Om två sådana överladdade toppar vandrar nära varandra bildas blandzoner med under-
liga topputseenden till följd vilket medför att det är svårt att veta när man ska börja och sluta fraktionera för att få rena substanser. Därför är preparativ kromatografi mycket svårare att optimera än analytisk. Men med kunskap om ämnenas adsorptionsisotermor, som visar sambanden vid konstant temperatur mellan koncentrationen av ämnet i mobila fasen och den på stationära fasen, kan man förutsäga de överladdade topputseendena.


Enbart koldioxid är ett dåligt lösningsmedel för många polära föreningar varför man idag i stor utsträckning blandar in mindre mängder polärt organiskt lösningsmedel som metanol eller etanol i den mobila fasen. Det faktum att det kromatografiska experimentet kan utföras snabbare innebär att analyser kan genomföras på kortare tid och att produktiviteten, d.v.s. mängd substans renad per tidsenhet kan ökas. Koldioxid som mobil fas ger också enklare uppsamling av de värdefulla fraktionerna rent ämne, i huvudsak eftersom all koldioxid helt enkelt förgasas vid atmosfärstryck eller återvinns.

Trots att det finns mycket empirisk kunskap kring SFC och att läkemedelsindustrin i stor utsträckning nyligen implementerat SFC för deras reningsprocesser av läkemedel, så saknas en djupare kunskap om SFC. Många decenniers forskning kring vätskekromatografi har resulterat i en god förståelse för de underliggande fysikaliska förloppen vilket har inneburit att man med matematiska modeller kan simulera och förutse hur de flesta förändringar av styrparametrar på-
verkar systemet. Med hjälp av numeriska simuleringar kan man opti-
mera det vätskekromatografiska systemet till att prestera optimalt. I
SFC kan man i många fall inte ens sätta upp en enkel modell som kan
beskriva hur systemet förändras då man ändrar pumpflödet. Kun-
skapsbristen inom SFC beror framförallt på att mobila fasen i överkri-
tiskt tillstånd är komprimerbar till skillnad från en vätska. SFC är som
en slags elastisk gummivariant av vätskekromatografi. I vanlig väts-
kekromatografi vandrar lösningsmedlet genom kolonnen med jämn
hastighet men i SFC får man gradierter när det gäller tryck, densitet,
temperatur och hastighet. Tillsammans påverkar dessa gradierter se-
parationsprocessen på ett mycket invecklat sätt.

Syftet med mitt avhandlingsarbete har varit att undersöka flera
grundläggande aspekter av SFC. Förutsättningen för detta har varit
att noggrant mäta och/eller beräkna parametrar såsom tryck, tempe-
ratur, flöden, densiteter och olika kompositioner av mobila fasen i ett
kommersiellt SFC instrument. Den senaste generationens SFC system
ger en begränsad information om hur dessa parametrar varierar och
ger därför dåliga förutsättningar för reproducerbara studier. Resulta-
ten av mina forskningsrön redovisades i två vetenskapliga publikation-
er (Artikel I och II). I huvudsak visade det sig att de metoder för
bestämning av adsorptionsisotermer som ger högst noggrannhet i
vätskekromatografi, så kallade platåmetoder, fungerar dåligt för SFC.
Istället rekommenderas metoder som normalt har sämre noggrannhet
i vätskekromatografin som baseras sig på data från överladdade kro-
matogram.

Därefter sattes statistiskt baserad försöksplanering upp ("Experimen-
tell design") för att ta reda på den relativa betydelsen av de olika expe-
imentella parametrarna och inställningarna på instrumentet för
bästa tänkbara separation, såväl analytisk som preparativt. Resultaten
kunde bekräfta tidigare observationer såsom att metanolhalten följt
av trycket visade sig vara de relativt viktigaste parametrarna för att
styra retentionen i två modellsystem. Temperaturen var viktigast för
selektiviteten. För att öka produktiviteten var en större andel metanol
och högre temperatur viktigt (Artikel III).
Artikel IV fokuserade på bakomliggande orsaker till toppdeformering i SFC, vilket allvarligt begränsar hur stor volym man kan injicera av provet vid SFC, till skillnad mot vätskekromatografi. Den dominerade metoden att injicera prov i SFC är i vätskeform, precis som vid vätskekromatografi. Detta innebär att man injicera en plugg av 100 % polärt organiskt lösningsmedel (i vätskeform) in i en mobilfas som huvudsakligen består av koldioxid, i överkritiskt tillstånd. Denna stora skillnad leder till att molekylerna i provlösningen upplever en kraftig jämviktstörning mellan mobil och stationärfas vilket man tidigare visat ge kraftiga toppdeformationer. Studien kunde både kvalitativt och kvantitativt genom simuleringar förklara uppkomsten av toppdeformationarna. Denna information är speciellt viktig för preparativ SFC eller då större volymer måste injiceras.

En av vätskekromatografins fördelar jämfört med SFC, är att det är mycket enkelt att skala upp ett analytiskt småskaligt experiment till preparativ skala. Det är precis tvärtom vid SFC och jag ville försöka komma tillräcka med detta problem i Artikel V. Jag använde ett enkelt separationssystem som modell och studerade vad som krävdes för att skala upp detta från en kolonn med 4.6 mm inre diameter till en motsvarande 50 mm kolonn. Studien genomfördes på ett analytiskt system på universitetet och ett storskaligt instrument på Astrazeneca i Mölndal. En nyckel till att lyckas med denna metodöverföring var att först noggrant karaktärisera flöden, tryck och temperaturer i det analytiska systemet och sedan överföra dessa i enheter som användes av det preparativa systemet.
5. Acknowledgement

This thesis would not have been possible without the inspiration, ambition and persistent help of many others. I would especially like to thank the following:

Dr. Torgny Fornstedt for inspiring me to endeavor in the field of chromatography. You have showed me that “good enough” is not good enough when it comes to research.

Dr. Jörgen Samuelsson for your endless knowledge in anything related (or not) to chromatography. Thanks for pushing me to continuously adopt a more thorough approach to my studies, and of course, for converting me from Ubuntu to Arch Linux.

Dr. Patrik Forssén for help with calculations and excellent eye for detail in the writing of our joint papers.

Dennis Åsberg for all help and great contributions to my studies. Also, for a very nice time during our stay in Uppsala.

All colleagues at Karlstad University and Uppsala University. Torgny Undin and Lena Edström for a nice collaboration during my first year as a PhD student. Mikael Andersén for always helping me with course work preparations.

Magnus Klarqvist, Hanna Leek, Kristina Öhlén for excellent support and contributions to the scale-up study during my stay at AstraZeneca, Mölndal.

Dr. Olle Gyllenhaal for giving me my first experience with SFC and for introducing me to the SFC community at the SFC conference in Brussels 2012.

Dr. Joakim Högbom for helping me start up the SFC research using the “should be placed in the attic and forgotten” instrument.
Dr. Arvind Rajendran for continuous feedback on my research during our meetings in Sweden, Europe and the U.S. Your early work in SFC guided me in the right direction.

Dr. Terry A. Berger for letting me know that I was on the right track in my research.

Dr. Robert Arnell for showing me how large scale chromatography works and how fundamental research can be applied to improve such processes.

Dr. Krzysztof Kaczmarski for quick, thoughtful and always helpful support. Of course also for traditional polish food and oil field sightseeing in southern Poland.

Dr. Abhijit Tarafder for a lot of helpful feedback to my research. Also thank you for the facility tour in Milford.

Dr. Fabrice Gritti for always asking the difficult but necessary questions during my “lecture series on SFC in Boston 2013 and 2014.

Dr. Georges Guiochon for some interesting and rewarding discussions on my research. The opportunity to read the proof of an early version of your seminal SFC review made me realize what fun lay ahead.


The Swedish Research Council and the Swedish Knowledge Foundation for generous grants supporting the research effort.

My family, friends for all your support. The foundation for this journey was laid long before the start of my PhD.

Finally, Sanne, even though we now both live on new continents, I love you more than ever.
7. List of References


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Fundamental Investigations of Supercritical Fluid Chromatography

This thesis aims at a deeper understanding of Supercritical Fluid Chromatography (SFC). Although preparative SFC has started to replace Liquid Chromatography (LC) in the pharmaceutical industry - because of its advantages in speed and its less environmental impact - fundamental understanding is still lacking. Therefore there is no rigid framework to characterize adsorption or to understand the impact of changes in operational conditions.

In Paper I-II it was demonstrated why most methods applied to determine adsorption isotherms in LC could not be applied directly for SFC. Methods based on extracting data from overloaded profiles should be preferred.

In Paper III a Design of Experiments approach was successfully used to quantitatively describe the behavior of several solutes in a separation system. This approach can be used to optimize SFC separations or to provide information about the separation system.

In Paper IV severe peak distortion effects often observed in SFC were carefully investigated and explained using experiments and simulations.

Finally, in Paper V, the prerequisites for performing reliable and predictable scale-up of SFC were investigated by small and large scale experiments.