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From on-line measurements to inverse modeling

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Fundamental research on supercritical fluid extraction kinetics:

From on-line measurements to inverse modeling

Victor Abrahamsson

LUND UNIVERSITY

Doctoral Thesis

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Abstract
Supercritical fluid extraction is an extraction technique suitable for lipophilic compounds from solid samples. Most commonly supercritical carbon dioxide is the main component in the extraction phase, rendering the technique relatively environmentally benign. The extraction technique is rapid due to the low viscosity and the high diffusivity of analytes in the supercritical extraction phase.

The selectivity can be tuned by changing the extraction conditions of pressure, temperature and co-solvent amount. These process parameters along with flow rate and extraction time make optimization of an extraction method rather cumbersome. A fundamental understanding of the extraction process can help to make wise decisions during method development. In this work extractability, partitioning, solubility and internal and external mass transfer resistance have been studied through inverse modeling.

Methods based on in-line spectrophotometric measurements and on-line evaporative light scattering detection have been developed to efficiently acquire extraction curves, i.e., the extraction yield over time. These enable a high-throughput of extractions with high temporal resolution and good precision. The methods were applied to quantify total lipids from linseed and carotenoids, chlorophyll A, ergosterol and total lipids from microalgae. An off-line method for separating carotenoids based on supercritical fluid chromatography was also developed.

Methodologies have been developed to acquire models which are calibrated using all experiments, so called complete calibration. It is shown that calibrating one model per experiment does not generate models with reliable parameters with physical meaning. The models can be used for predicting extraction curves within the investigated space of process parameters.

Finally, extractability and partitioning are shown to be highly influential on the extraction process. Also, partitioning can give rise to diminishing extraction rates, which has previously believed only to be caused by intra-particle diffusion.

Key words
Supercritical fluid extraction, supercritical fluid chromatography, linseed, microalgae, extraction kinetics, inverse modeling, curve resolution

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Populärvetenskaplig sammanfattning

Provupparbetning är ett viktigt steg i många metoder för kemisk analys. Detta gäller framförallt fasta prover då de kemiska ämnena som avses för analys, även kallade analyter, måste extraheras ut. Det vill säga att genomföra en överföring av analyter från provet till ett lösningsmedel. Därefter kan halten bestämmas med de metoder som ofta finns till hands i ett analyslaboratorium. I de fall då fettlösliga analyter är av intresse krävs oftast organiska lösningsmedel för extraktion. Dessa är tämligen besvärliga då de ofta är hälsofarliga, brandfarliga samt skadliga för miljön. Utöver detta medför de även en kostnad vid inköp och vid destruktion. Ett miljövänligare alternativ är att använda koldioxid som lösningsmedel, som även är billigare och relativt ofarligt.

Genom att trycksätta och värma koldioxiden kan den superkritiska punkten nås. Mediet får då egenskaper som är mittemellan en vätska och en gas. Framförallt är låg viskositet och snabb diffusion åtråvärda då analyterna kommer att extraheras i en snabbare takt. Traditionella extraktionsmetoder med organiska lösningsmedel kan ta en hel arbetsdag att genomföra medan med superkritisk koldioxid kan extraktionstiden kortas ned till cirka en timme. Eftersom koldioxiden försvinner som en gas vid atmosfäriskt tryck behövs heller ingen indunstning av provet, vilket sparar ytterligare tid.

Denna avhandling behandlar fundamentala aspekter kring hur denna process fortgår. Processparametrar som till exempel tryck, temperatur, inblandning av alkoholer, flöden och extraktionstid har inverkat på hur stor andel av analyterna som blir extraherade och till vilken hastighet. De bakomliggande faktorerna är de som har direkt inverkan. Dessa är löslighet, fördelning mellan det fasta provet och den superkritiska vätskan, diffusion inom provet och diffusion genom den stagnanta film som bildas kring en partikel vid ett flöde kring den. Analyterna kan också vara otillgängliga ifall de till exempel har blivit adsorberade till det fasta provet.

Dessa processer kan tyvärr inte direkt studeras eftersom extraktionen

sker i en trycksatt behållare, och nästan alltid i över 100 atmosfäriskt tryck. Här presenteras därför flera metoder för att kunna mäta koncentrationshalten av analyter kontinuerligt i utflödet. Därefter har matematiska metoder, så kallad inversmodellering, utvecklats för att med hjälp av den experimentella datan kunna indirekt studera de processer som påverkar extraktionshastigheten. Den grundläggande kunskapen som erhålls är till stor nytta för att förstå och kunna optimera denna provupparbetningsteknik. Till exempel, tidigare studier har poängterat att det är framförallt diffusion inom provet som orsakar en avtagande extraktionshastighet med tiden. I detta arbete argumenteras det för att det likväl kan vara på grund av partitionering mellan provet och den superkritiska vätskan.

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals. The papers are appended at the end of the thesis.

I **Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography**
Victor Abrahamsson, Irene Rodriguez-Meizoso, Charlotta Turner
Journal of Chromatography A, **1250**, 63-68 (2012)

II **Supercritical fluid extraction of lipids from linseed with on-line evaporative light scattering detection**
Victor Abrahamsson, Irene Rodriguez-Meizoso, Charlotta Turner
Analytica Chimica Acta, **853**, 320-327 (2015)

III **Method development in inverse modeling applied to supercritical fluid extraction of lipids**
Victor Abrahamsson, Niklas Andersson, Bernt Nilsson, Charlotta Turner
Journal of Supercritical Fluids, **111**, 14-27 (2016)

IV **Continuous multi-component detection in supercritical fluid extraction applied to microalgae using inline UV-Vis spectroscopy and on-line evaporative light scattering detection**
Victor Abrahamsson, Firas Jumaah, Charlotta Turner
Submitted

V **Multicomponent inverse modeling of supercritical fluid extraction of carotenoids, chlorophyll A, ergosterol and lipids from microalgae**
Victor Abrahamsson, Larissa Cunico, Niklas Andersson, Bernt Nilsson, Charlotta Turner
Manuscript

List of Contributions

I VA: Involved in the planning, performed all of the experiments and wrote the manuscript with support.

IRM: Involved in initial experiments, involved in the planning and revised the paper.

CT: Involved in the planning and revised the paper.

II VA: Synthesized the initial research idea, performed all of the planning, experiments, data evaluation and wrote the manuscript.

IRM: Revised the manuscript.

CT: Involved in the planning and revised the manuscript.

III VA: Performed all of the planning, experiments, modeling, data evaluation and wrote the manuscript.

NA: Involved in the planning, supported with modeling expertise, helped running simulations and revised the paper.

BN: Involved in the planning and revised the paper.

CT: Involved in the planning and revised the paper.

IV VA: Synthesized the initial research idea, performed all of the planning, performed extraction experiments, most of the off-line analysis, data evaluation and wrote the majority of the manuscript.

FJ: Performed the supercritical fluid chromatography analysis and revised the paper.

CT: Involved in the planning and revised the manuscript.

V VA: Performed all of the planning, experiments, modeling, data evaluation and wrote the manuscript.

LC: Performed density calculations, supported with solubility modeling expertise and revised the manuscript.

NA: Involved in the planning, supported with modeling expertise, helped running simulations and revised the manuscript.

BN: Involved in the planning and revised the manuscript.

CT: Involved in the planning and revised the manuscript.

List of paper not included in this thesis

VI **Determination of sulfite in beer based on fluorescent derivatives and liquid chromatographic separation**
Victor Abrahamsson, Signe Hoff, Nikoline J. Nielsen, Marianne N. Lund, Mogens L. Andersen
Journal of the American Society of Brewing Chemists, **70**, 296-302 (2012)

VII **Extraction and neoformation of antioxidant compounds by pressurized hot water extraction from apple byproducts**
Merichel Plaza, Victor Abrahamsson, Charlotta Turner
Journal of Agricultural and Food Chemistry, **61**, 5500-5510 (2013)

VIII **Impact of injection solvents on supercritical fluid chromatography**
Victor Abrahamsson, Margareta Sandahl
Journal of Chromatography A, **1306**, 80-88 (2013)

IX **A fast and sensitive method for the separation of carotenoids using ultra-high performance supercritical fluid chromatography-mass spectrometry**
Firas Jumaah, Merichel Plaza, Victor Abrahamsson, Charlotta Turner, Margareta Sandahl
Analytical and Bioanalytical Chemistry, **408**, 5883-5894 (2016)

X **Comprehensive two-dimensional gas chromatography in combination with pixel-based analysis for studying and predicting fouling tendencies of gas condensates in a steam cracker reactor**
Victor Abrahamsson, Nenad Ristic, Kristina Franz, Kevin Van Geem
Submitted

Abbreviations

2-EP 2-ethyl pyridine

BIC broken and intact cells

BPR backpressure regulator

CCD charged-coupled device

CDF common data format

CLS classical least squares

CO_2 carbon dioxide

CSTR continuous stirred-tank reactor

csv comma-separated values

DAD diode array detector

DLT diffusion layer theory

DOE design of experiments

ELSD evaporative light scattering detection

FIM Fischer information matrix

FVM finite volume method

GA genetic algorithm

GC gas chromatography

HBM hot ball model

HPLC high-performance liquid chromatography

LC liquid chromatography

LHS Latin hypercube sampling

MADS mesh adaptive direct search

MAE microwave assisted extraction

MCR multivariate curve resolution

MCR-ALS multivariate curve resolution alternating least squares

MS mass spectrometry

ODEs ordinary differential equations

PARAFAC parallel factor analysis

PARAFAC2 parallel factor analysis 2

PAT process analytical technology

PCA principal component analysis

PDEs partial differential equations

PLE pressurized liquid extraction

PSO particle swarming optimization

RMSE root mean square error

RSM response surface methodology

$scCO_2$ supercritical carbon dioxide

SCF supercritical fluid

SFC supercritical fluid chromatography

SFE supercritical fluid extraction

SLE solid-liquid extraction

SVD singular value decomposition

UAE ultrasound assisted extraction

UHPSFC ultra high-performance supercritical fluid chromatography

Table of Contents

Chapter 1

Introduction

> *"Begin at the beginning," the King said, gravely, "and go on till you come to an end; then stop."*

Lewis Carroll, *Alice in Wonderland*, 1899

1.1 Background

The general process flow of which an analytical chemist works according to does in general not start with an explicit hypothesis emerging from analytical chemistry [Harris, 2007]. More often the initial question emerges from a different scientific domain. For example might the environmental scientist wonder how contaminated the water is. Policy makers, managers and scientists may need to make qualified decisions based on chemical measurements.

The general steps of chemical analysis can bluntly be categorized into sampling, sample preparation, chemical analysis and data analysis. The increasing needs for quantifying at lower concentrations, compounds which have never been analyzed before, with better trueness and precision, and with quicker total analysis time is what drives analytical chemistry forward. However, in hindsight it is safe to say that most emphasis has been directed towards chemical analysis. Hedrick et al. [1992] simply states that not only is sample preparation the most time-consuming step, but it is also the most error-prone, least glamorous and most labor intensive task in the laboratory. It therefore becomes crucial to emphasize that no chemical analysis or statistical methodology can ever compensate for a poor sampling procedure

or an incorrect sample preparation step. The results and conclusions can easily become meaningless even unknowingly so.

Furthermore, as the instrumentation has progressed immensely over the last decades and especially considering chromatography and mass spectrometry, the main bottleneck in terms of time may often be the sample preparation step. One should also consider that nowadays the sample preparation, particularly extraction of non-polar compounds, often require more organic solvents than any subsequent step involving e.g., liquid chromatography [Tobiszewski et al., 2009]. By either reducing, replacing or recycling the organic solvents used in the sample preparation step, the environmental impact of the overall analysis method can be considerably diminished [Welch et al., 2010]. Reducing, replacing and recycling is also known as the three Rs and is a widely used concept in the context of green chemistry.

In summary analytical chemistry faces some key challenges which can be addressed by fundamental research in sample preparation. The sample preparation methodology should preferably strive to be the following:

- Rapid
- Free from bias, namely good trueness
- Have a high precision
- Have a low environmental impact
- Of low cost
- Simple

In extraction and analysis of hydrophobic compounds from solids, supercritical fluid extraction (SFE) provides a viable alternative to the conventional and time consuming Soxhlet extraction technique [Hedrick et al., 1992]. By utilizing carbon dioxide (CO_2) as a solvent and bringing it to supercritical conditions by maintaining a high pressure and applying slight heating, the technique becomes rapid due to efficient mass transfer and the environmental impact is reduced due to the replacement of organic solvents [Brunner, 1994].

Although SFE has shown great potential over the years considering the very diverse applications, there are still many fundamental aspects of the technique which are in need of further investigation. As the title of this thesis suggests, the main topic of interest in this work is to develop a deeper understanding of the kinetics and mass transfer phenomena of SFE. Much

effort has been given in developing novel methods in order to acquire experimental data and to convert the acquired data into new knowledge of the actual underlying phenomena taking place in the SFE process.

The research conducted in this work may very well benefit already established areas of applications. Examples of such are sample preparation in the analytical laboratory or industrial applications like decaffeination of coffee beans [Clifford, 1999]. The outlooks of SFE as an extraction technique in pharmacognosy are experiencing a positive trend [Cheng et al., 2001, Kataoka, 2010]. This refers to extraction of pharmaceuticals from plant material. By understanding the extraction process in greater depth more selective methods can be developed. The knowledge can also be used for determining the influence of the extraction conditions on the purity [Brueggemeier et al., 2012].

1.2 Solid sample preparation in general

Among the many various sample matrices faced by the analytical chemist, e.g., air, water, body fluids, the analysis of solid materials presents a formidable challenge. For example, in the analysis of liquids the analyst can in many instances get away with a *dilute-and-shoot* methodology [Deventer et al., 2014]. However, a solid sample cannot be diluted and directly injected into e.g., a liquid chromatography system. In many cases a solid sample can simply not even be dissolved into a solvent, and although it might be possible, the acquired solution may be too complex for subsequent chemical analysis [Švarc-Gajič, 2012].

By employing a selective sample preparation method, the problem at hand can be greatly simplified. Extraction of analytes from a solid matrix, also known as leaching, is often an overlooked step in the chain of analytical chemistry. Several considerations should be kept in mind. Solid systems are typically heterogeneous, complex and analytes are subdued to matrix effects which are in general impossible to predict and largely deviating between sample matrices [Švarc-Gajič, 2012]. These matrix effects may be caused by for example chemical bonding between the analyte of interest and the solid matrix, or by a mechanical barrier hindering the solvent to reach the space where the analyte is distributed [Vazquez-Roig and Picó, 2015].

Other limiting effects could be based on kinetics and mass transfer phenomena. The partitioning of the analyte between the solid sample and the extraction solvent is an example of kinetics which effects the extraction to a great extent [Clifford, 1999].

Typical sample preparation techniques used for extraction from solid matrices are e.g., solid-liquid extraction (SLE), pressurized liquid extraction (PLE) [Björklund et al., 2000], microwave assisted extraction (MAE) [Luque-García and Luque De Castro, 2003a], ultrasound assisted extraction (UAE) [Luque-García and Luque De Castro, 2003b] and SFE [Clifford, 1999]. Among these sample preparation techniques, perhaps the most commonly used and also the technique with the longest history is the SLE using the Soxhlet apparatus. The Soxhlet method may require an extraction time up to 24 h in order to achieve a complete extraction from a solid material [Snyder et al., 1992]. The long extraction time can be circumvented by increased mass transfer rates through higher diffusion rates and lower viscosity by utilizing higher temperatures as by the principles of PLE and MAE [Švarc-Gajič, 2012]. UAE benefits from increased mass transfer rates prominently by inducing irregular flow through the formed accustic cavities, which reduces the stagnant layer surrounding each particle, through which analytes must diffuse [Esclapez et al., 2011]. SFE is also favored by increased mass transfer rates due to inherent lower viscosities and higher diffusivity, even at relatively low temperatures [Clifford, 1999]. This is beneficial in scenarios where heat sensitive compounds are to be extracted. Furthermore, SFE can also be combined with UAE in order to further improve extraction times [Gao et al., 2009, Riera et al., 2010].

The above-mentioned techniques have their merits, however, besides SFE they in general require non-polar organic solvents for the extraction of e.g., lipids. By employing CO_2 as a solvent these expensive and hazardous organic solvents can be avoided. Furthermore, subsequent evaporation steps are avoided or minimized as the CO_2 is quickly removed after the depressurization.

1.3 Aim of the thesis

The grand aim of this thesis is to study the fundamentals of SFE kinetics. The scientific endeavor has been directed towards a series of objectives.

- Develop necessary off-line analysis methods in order to study collected fractions.
- Develop on-line measuring methodology for studying extractions curves, i.e. extraction yield over time.
- Develop necessary methodology for performing inverse modeling of SFE processes based on extraction curves.

- Utilize developed methods to identify and study the phenomena which govern the SFE process.

1.4 Outline of the thesis

Although analytical chemistry is already a very interdisciplinary research field, this thesis incorporates many ideas and concepts from the field of chemical engineering regarding e.g., mass transfer phenomena in packed beds or from the field of chemometrics e.g., signal processing. The included papers reflect this bridging, however, they have in some cases been directed towards a specific audience within a certain field of research. In this thesis attention is given to arch the gap between the various fields but not necessarily to describe all concepts in depth in order to maintain a natural continuation between the topics.

This thesis could also be regarded as an introduction or a tutorial on how to study kinetics of SFE processes based on so called extraction curves. The proposed workflow is presented in Figure 1.1. Attention will be given to practical aspects as well. In addition to an introduction of supercritical fluid and the SFE process, each of the steps: experimental measurements, data processing, inverse modeling and design of experiments are represented by their own chapter. Although it might be counter-intuitive, design of experiments is discussed last, since understanding the model structure is essential before the most suitable experiments can be decided upon. In the grand scheme, the problem is truly a catch 22.

Chapter 2: Supercritical fluid extraction

A short introduction is given to supercritical fluids and why they are particularly useful. A brief overview of SFE is also given.

Chapter 3: Experimental measurements

Previous studies found in the literature mainly focus on performing analysis off-line by collecting fractions. On-line measurement techniques were applied in order to minimize analyte losses during sample collection, errors due to additional sampling handling and time-consumption. In **Paper II** evaporative light scattering detection was evaluated and validated for real-time continuous measurement of lipids extracted from crush linseed. In **Paper IV**, the instrumental system was further developed by also using in-line UV/Vis spectrophotometry in combination with SFE applied to microalgae.

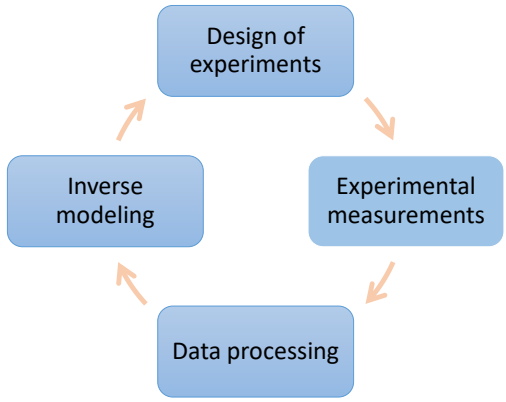

Figure 1.1: Schematic of the workflow proposed for studying the governing phenomena of SFE. Any of the blocks can be a starting point of a study, or a series of studies.

Additionally, an analysis method was developed in order to rapidly separate and quantify individual carotenoids from microalgae in **Paper I**.

Chapter 4: Data processing

It can be challenging to manage the acquired data from the many needed experiments. In **Paper IV**, a methodology for signal processing is proposed and an evaluation of various curve resolution techniques including classical least squares, multivariate curve resolution-alternating least squares and parallel factor analysis. These techniques enable deconvolution of the acquired landscape of the spectrophotometric measurements representing wavelength, extraction time and signal intensities, into pure analyte spectra and relative analyte concentrations. Hence, individual extraction curves can be acquired for each studied analyte.

Chapter 5: Inverse modeling

Inverse modeling allows for an indirect approach to study underlying phenomena which cannot be measured directly. Parameters reflecting physical properties can be derived out of measured extraction curves. A methodology was developed based on experimental data of lipid extraction from crushed linseed in **Paper III**. In **Paper V** the extraction kinetics of carotenoids, chlorophyll A, ergosterol and total lipids from microalgae were studied.

Chapter 6: Design of experiments

The design of experiments in the context of studying underlying SFE kinetics presents a challenge. In **Paper III** a classical full-factorial design based on the process factors was used. In **Paper V** the experimental design was based on the parameters density and temperature, rather than pressure and temperature. The impacts and possible further developments are discussed in this chapter.

Chapter 7: Conclusions

A capitalization of the obtained results is presented in a brief summary. Finally, a short open discussion is given concerning potential future work.

In addition to forming the glue which holds the included scientific papers together, additional data, findings and reflections which were deemed out of scope for each individual paper or which have been derived based on newly gained knowledge is presented throughout this thesis.

It is recommended that the reader with moderate or less experience and knowledge of SFE, after reading this introduction chapter, start in logical order with Chapters 2 and 3 accompanied by **Papers I-II**. It then naturally follows to get acquainted with Chapter 4 and **Paper IV**. The reader should then continue on to read Chapter 5 and **Papers III and V**, and finally finish with Chapter 6.

Each of the chapters can of course be read individually depending on previous knowledge and interests.

Chapter 2

Supercritical fluid extraction

> *Chemistry begins in the stars. The stars are the source of the chemical elements, which are the building blocks of matter and the core of our subject.*

Peter Atkins

2.1 Supercritical fluids

A supercritical fluid (SCF) will be attained by bringing a pure component, or a mixture, beyond its critical values of pressure and temperature (Figure 2.1). Although any substance can be in its supercritical state, relatively many could in theory be used in various applications, only a few substances are actually utilized in practice. Many substances in practice degrade before reaching the supercritical state or are not suitable due to toxicity or the risk of explosions. In analytical chemistry and in extraction, CO_2 is almost exclusively used, with or without a so called co-solvent [Brunner, 1994, Janda et al., 1993, Levy, 1999]. The co-solvent is used to alter the polarity of the mixture. CO_2 is beneficial because it is inert, non-toxic, readily available and cheap. Therefore, this thesis is limited to systems where CO_2 is the major component. In a binary system the critical temperature and the critical pressure of the mixture will depend on composition and each of the components critical values. Some typically used gases and liquids are presented in Table 2.1.

It is worth pointing out that the conditions inside the supercritical fluid chromatography (SFC) separation column are rarely supercritical. This is a

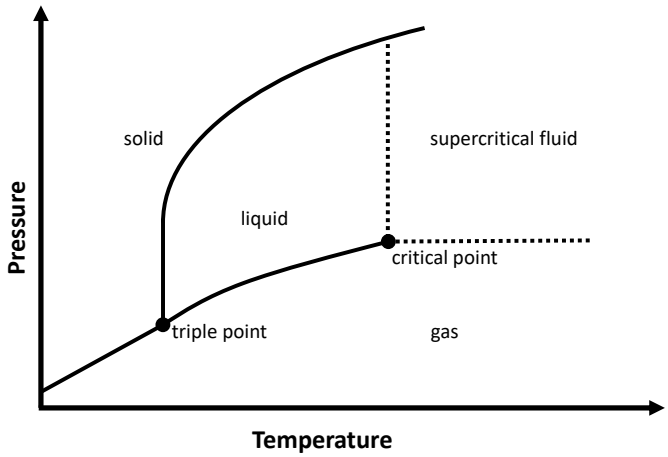

Figure 2.1: Example of a phase diagram for a pure component.

Table 2.1: Critical data for some pure components

Component	T_c (K)	P_c (MPa)
Carbon dioxide	304.15	7.38
Ethane	305.4	4.88
Propylene	364.95	4.60
Methanol	512.6	8.09
Ethanol	513.9	6.14
Isopropanol	508.3	4.76
Water	647.1	22.06

result of the high fractions of co-solvent, which is usually an alcohol [Lesellier and West, 2015], in combination with the low operating pressures. Typically, SFC is operated at temperatures between 40 and 60 °C [Lesellier and West, 2015, Nováková et al., 2014]. For example, the conditions of the SFC in **Paper 1** were rather sub-critical.

Many properties of a supercritical fluid are interesting and deviate substantially from liquids at ambient conditions. Perhaps the most interesting properties in the context of SFE are solubility [Škerget et al., 2011], and the two well-correlated properties viscosity and diffusivity [Magalhães et al., 2013]. As a simple rule-of-thumb, it can be said that the properties of a SCF is between those of a gas and a liquid. Meaning that the beneficial mass transfer properties is achieved through higher diffusivity and lower viscosity, while on the other hand, the density is somewhat lower as compared

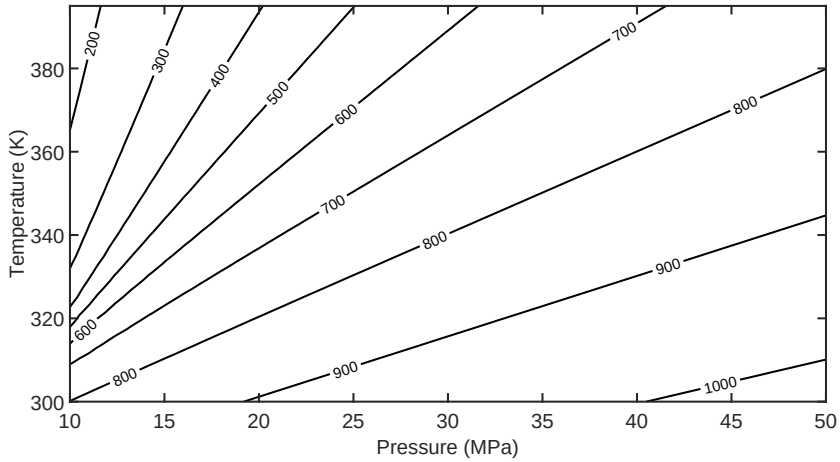

Figure 2.2: Contour lines visualizing the influence of pressure and temperature on the density (g/L) of $scCO_2$. The data was retrieved from the NIST Chemistry WebBook [Lemmon et al., 2016].

to a traditional liquid solvent. These properties are anyhow highly dependent on the process parameters pressure and temperature. The density and viscosity for pure supercritical carbon dioxide ($scCO_2$) at various pressures and temperatures are given in Figure 2.2 and Figure 2.3, respectively.

The $scCO_2$ is in general seen as similar to e.g., hexane in terms of polarity. It should be recognized that although $scCO_2$ has a low dielectric constant and zero molecular dipole moment, it has a substantial quadruple moment and is capable of acting as both a weak Lewis acid and Lewis base [Raveendran et al., 2005]. In practice the solubility of analytes in pure $scCO_2$ can be tuned by changing the pressure and the temperature. This affects the vapor pressure of the solute and solvent molar volume, i.e., the density of $scCO_2$ [Škerget et al., 2011]. At a constant pressure, an increase of temperature will decrease the density. Overall, both increased density and vapor pressure of the solute results in a higher solubility [Clifford, 1999]. The relationship between density, temperature and solubility is not easily predicted. The solubility can be estimated through e.g., Peng-Robinson equation of state. Many parameters are needed and must be estimated experimentally, whereof these increase with the number of components in the system [Clifford, 1999]. In practice in the research field of SFE, simple empirical correlations based on density and temperature alone are often used [Škerget et al., 2011], which is discussed in Chapter 5.

The diffusivity, which is a key factor upon describing mass transfer phe-

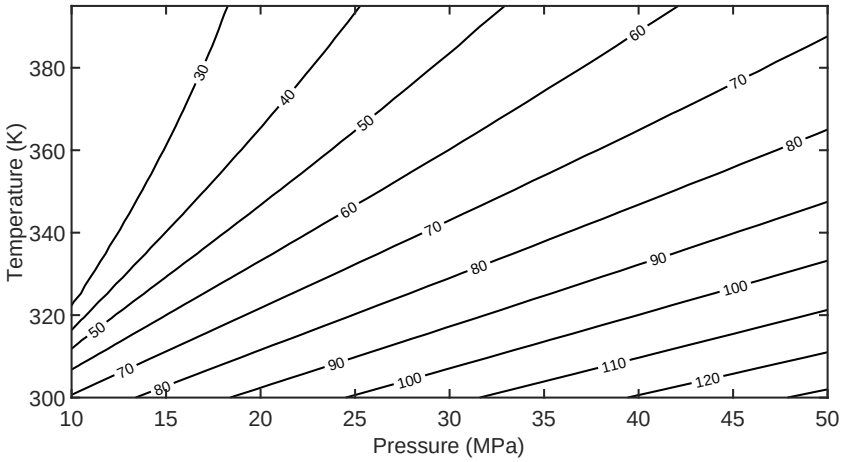

Figure 2.3: Contour lines visualizing the influence of pressure and temperature on the viscosity (μPa $\cdot$ s) of $scCO_2$. The data was retrieved from the NIST Chemistry WebBook [Lemmon et al., 2016].

nomena is correlated with temperature, density and viscosity, which in turn correlate among themselves. A denser system has lower diffusion rates and a higher temperature results in higher diffusivity [Funazukuri et al., 2006]. The binary diffusion (one analyte in a solvent) can be correlated by using either exclusively viscosity; temperature and viscosity; temperature and density; or temperature, density and viscosity [Magalhães et al., 2013]. These simple but accurate correlations suggested by Magalhães et al. [2013] were also applied in **Paper III**.

The addition of a co-solvent, also called entrainer or modifier, is usually performed to increase the polarity of the otherwise non-polar $scCO_2$. It is, however, important to be aware of the effects such an addition will have on e.g., density and viscosity [Clifford, 1999, Tarafder and Guiochon, 2012].

2.2 The apparatus

SFE is an important industrial scale process, as well as a technique in analytical chemistry. In either case the basic principles of the process are described in figure 2.4. The fluid, usually CO_2, is pumped as a liquid and is therefore cooled. The liquid CO_2 is much less compressible than in its gaseous or supercritical state and is thus more readily pumped [Lemmon et al., 2016]. The cooling is applied directly on the reservoir on an industrial

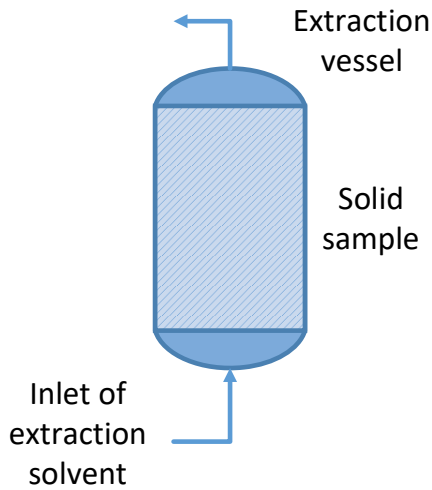

Figure 2.4: A simple schematic of a SFE process. The solid sample is placed inside the extraction vessel.

or on a pilot plant scale, while on a small scale in an analytical chemistry laboratory the cooling of the pump head is sufficient [Clifford, 1999]. An additional pump may be added for the addition of a liquid modifier. The fluid is subsequently heated before entering the extraction vessel of which the temperature is also controlled. On a small scale this is performed by either mounting a coil of tubing followed by the extraction vessel inside a temperature controlled oven, or by using a heated block unit where the extraction vessel is mounted. In this work a gas chromatography (GC) oven was used for controlling the temperature. The heating along with the pressure will bring the solvent into the supercritical region as was described in Figure 2.1.

The extraction vessel, which holds the sample matrix, is equipped with frits in both ends in order to ensure that the sample matrix itself is not pushed out of the extraction vessel. The extraction vessels used are similar to columns used in high-performance liquid chromatography (HPLC). Although empty HPLC columns can be used as extraction vessels, we have noticed that these seldom last because of leakage due to frequent opening and closing. Leakage is a common practical problem when performing SFE. Based on our experience, fitting and refitting connections is in general only performed when mounting the extraction vessel and is therefore the most common location of leakage. If a leak is present, the experiment at hand

needs to be repeated with a new sample, since extracted analytes might have been lost through the leakage.

In order to achieve pressure in the system a capillary restrictor or a backpressure regulator (BPR) is positioned after the extraction vessel. The former was more frequently used in the past, whilst the latter is now implemented in most commercial systems. Capillary restrictors benefit from very small additional void volume to the SFE system. On the downside is high tendencies of clogging and blocking inside the capillary and that specific dimensions of capillary need to be used for each specific flow rate, modifier fraction and back-pressure. BPRs are in general based on a needle configuration which is either spring-loaded or automated with specific discrete positions into the socket. These are adjustable to achieve a stable and specific pressure at any set flow rate. The BPR, however, have a larger void volume which may introduce some lag-time between extraction and collection, although this is not a problem in most applications. The additional void volume could, however, cause problems of mass transfer as the $scCO_2$ expands into a gas and thus severely lowers the solubility. By employing a make-up flow this can be circumvented, which is discussed in Chapter 3.

More importantly, the BPR is equipped with seals that are in contact with the extraction fluid. The material of the seals may vary, but in general those materials used in commercial SCF applications degrade when using too high fraction of modifier. This may hinder flushing of analytes to the collection vial at the end of an extraction or cleaning of the SFE system by using e.g., pure alcohol. In this work, a spring-loaded BPR was exclusively used, due to its simplicity, price yet yielding more flexibility and robustness compared to a capillary restrictor.

Another consideration regarding the BPR is the required heating which needs to be applied. The expansion of the $scCO_2$ to gas is endothermic, thus heating is required in order to avoid freezing of the BPR and the following tubing. However, applying too much heating could possibly cause degradation of heat sensitive compounds. Furthermore, it could also reduce the analyte transport after the depressurization point if a co-solvent or a make-up solvent is used. The reduced transportation from the BPR is likely cased by the evaporation of the co-solvent and the make-up solvent. This is further discussed in Chapter 3.

Collection can be performed in a series of pressurized collection vessels by step-wise pressure reduction through several BPRs [Brunner, 1994, Clifford, 1999]. This is typically performed on an industrial scale where for example fractionation can be achieved by pressure reduction. On a small scale the collection is typically performed at atmospheric pressure, with

or without a collection solvent in the collection vessel. This topic and its impact on actual chemical measurements is discussed more thoroughly in Chapter 3.

In any setup, the safety is always a concern due to the high pressure. Luckily, on a small scale typical for the analytical chemistry laboratory, small volumes are used. Meaning that the releasable energy is relatively low, as the relationship is proportional to the containment volume [Fryer and Harvey, 1997]. Nonetheless, it is important to incorporate rupture discs for each section of the equipment which may be closed during some part throughout the process. These ensure that if for example a clogging or an unexpected heat increase occurs, the system will not explode, but rather depressurize in a safe manner. This way of thinking is, however, only applicable when using an inert gas like CO_2. For example, Raynie [1993] reports and visualizes the consequences of using nitrous oxide, which is corrosive, where the extraction vessel exploded even though rupture discs were incorporated into the extraction system. In addition, it may be recommended to include one or several exhaust valves in case a manual depressurization is needed without needing to open the BPR valve.

In this work, non-continuous syringe pumps with an external cooling jacket with circulating cooling liquid were used. These are precise, however, based on own experience they may require up to half an hour to reach steady-state temperature inside the syringe upon refilling. Since the temperature influences the density, this is an essential aspect to achieve a correct flow rate. The additional time necessary to acquire a stable temperature lowers sample throughput and also hinders longer uninterrupted extractions. HPLC pumps were used as modifier and make-up solvent pumps. An old gas chromatograph oven was used as a controlled oven where the coiled tubing and the extraction vessel were housed. The volumes of the extraction vessels used typically ranged between 2 mL and 5 mL. A spring-loaded BPR was used, where the BPR and the following tubing was heated with a heating tape. The heating tape itself is extremely easy to apply and to use, however, the trueness of the temperature may be questionable. Simply because the temperature is measured in close proximity to the heating tape by a temperature sensor, however, this does not necessarily correspond to the temperature inside the capillary.

Besides the basic equipment, in-line and on-line detection was incorporated into the system based on UV/Vis and evaporative light scattering detection (ELSD) detection. This is more thoroughly discussed in Chapter 3. A picture of the assembled system based on the various stand-alone components is shown in Figure 2.5.

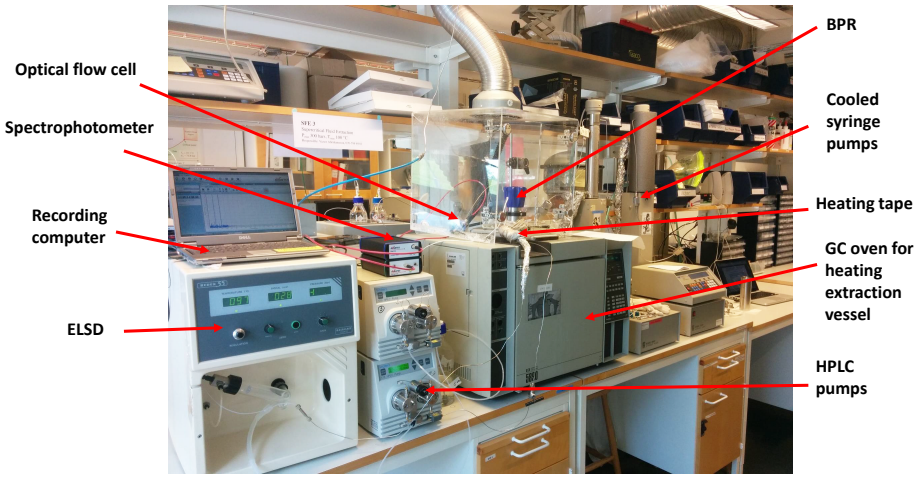

Figure 2.5: Picture of the SFE-UV/Vis-ELSD system used in this work.

2.3 The process

The process may be operated at various modes. The extractions are almost always performed in batch, although continuous processes have been reported, where the sample has been pumped as a liquid or a slurry [Karásek et al., 2003, Porta et al., 2011, Ryan and Stiver, 2007]. The SFE can be performed using static or dynamic extraction, or even a combination of the two. Static extraction refers to filling up the extraction vessel with the extraction solvent and maintaining the pressure without any flow, preferably until an equilibrium is achieved. The alternative, and perhaps the more commonly used approach, is the dynamic extraction where fresh extraction solvent is passed through the (immobilized) sample matrix. Dynamic extraction thus makes use of an increased analyte concentration gradient between the bulk fluid and the sample matrix [Brunner, 1994].

In any of the modes, the extraction rate and the converged extraction yield is governed by solubility, mass transfer phenomena (i.e., diffusion) and matrix effects. The SFE process is usually viewed as a packed bed with porous material (Figure 2.6. Although the concepts are briefly mentioned here, they are discussed in greater detail in Chapter 5.

The solubility is greatly governed by density, temperature and modifier fraction. The diffusion in SFE may be altered by changing the same parameters. However, the effective diffusion path length can easily be adjusted

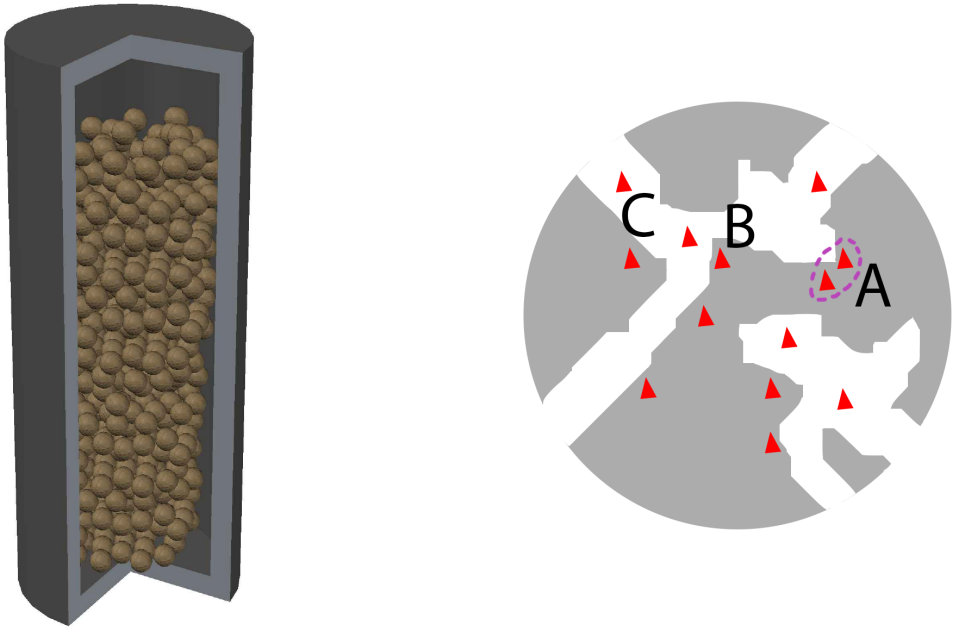

Figure 2.6: A schematic of a packed bed (left) consisting of porous particles (right). The analytes (red triangles) can be unextractable due to matrix effects (A). The extractable fraction will partition between the solid material and the extraction phase (B), which also depends on the solubility of the analyte in the extraction phase. The solubilized analyte will then diffuse out of the particle and into the bulk fluid (C).

by simply reducing the particle size by for example grinding. Care needs to be taken as excessive grinding of samples rich in lipids may generate a paste. The paste may not be efficiently exposed to the extraction solvents, and therefore a lower extraction yield may be acquired. This phenomenon was observed upon excessive grinding of already crushed linseed.

The above-mentioned phenomena mainly affect the extraction rate, assuming that at least the analyte of interest is partially soluble in the SCF. Matrix effects on the other hand mainly influence the point towards the SFE yield converges. Analytes must be released by the matrix if they are initially adsorbed or physically trapped by e.g., a cell wall or a polymer structure [Clifford, 1999]. The presence of water may reduce the availability and the extraction rate, particularly of very hydrophobic compounds [King et al., 1989, McNally, 1995]. In other cases the addition of water may actually assist the extraction efficiency by acting as a modifier [Mohamed et al., 2002]. This is for example typically done in the decaffeination process of coffee beans [Katz, 1989].

Channeling effects could possibly be mistaken for matrix effects, since it will generally lead to lower recoveries. Channeling effects are caused by heterogeneous flow velocities throughout a packed bed, which can occur at such an extent that a fraction of the sample matrix is not exposed to the extraction fluid [Sovová, 2006]. This is mainly an issue in leaching processes although viscous fingering is common when two solvents have substantially different viscosity [Kawaguchi et al., 1997, Shalliker and Guiochon, 2010]. When a packed bed is heterogeneous, the extraction fluid will preferably flow through the pathway with the least hydraulic resistance [Sovová, 2006]. This is especially true for samples which are sticky, moist or consist of very fine particles [Del Valle et al., 2012, Sovová, 2006]. In several published works, dispersion agents like silica beads or fine sand is added with the motivation that it removes channeling effects [Yamini et al., 2002]. The topic is further dissected and additional findings are discussed in Chapter 3.

As previously mentioned, the water content of a moist sample could be a problem. In many cases drying of the sample is performed [Clifford, 1999]. Alternatively, hydromatrix (diatomaceous earth) or other drying agents can be mixed with the sample before loaded into the extraction vessel [Burford et al., 1993]. Hydromatrix adsorbs excess water of the sample matrix, however, caution needs to be exercised as analytes might adsorb onto the drying agent [Turner et al., 2001].

2.4 Applications

SFE is of interest on all scales, as was mentioned earlier. On a commercial scale SFE is widely applied in processes related to the food industry. Operating costs are relatively low, although investments are steep, which makes SFE an attractive option. Some of the key advantages according to Sahena et al. [2009] are:

- CO_2 is completely free of organic solvents
- It is free from heavy metals and will not dissolve metal during the process either
- No salts are extracted
- No solvent removal is needed

Due to some of these facts, it does not need to be declared as an ingredient in food products [Brunner, 2005]. Commercial applications have focused mainly on high value compounds. The most famous applications

are the decaffeination of coffee beans [Katz, 1989] and of tea leaves [Joshi et al., 2013], as well as the production of hops extracts [Zeković et al., 2007]. Smaller industrial scale processes include extraction of spices, aroma and flavoring compounds, nutraceuticals and other bioactive compounds which are associated with positive human health effects [Brunner, 2005, Herrero et al., 2013, Sahena et al., 2009]. By no means is it feasible to mention all relevant original research publications which have applied SFE on various matrices in order to recover these compounds.

Nonetheless, much research has been conducted on acquiring oils from various seeds and bioactive compounds from microalgae. Oils acquired by SFE from e.g., cherry seed [Straccia et al., 2012], rose hip seed [Szentmihályi et al., 2002], coriander seed [Illés et al., 2000] and linseed [Chauhan et al., 2015] have all been shown to contain bioactive compounds, antioxidants or desirable essential oils. SFE applied to linseed has also been proposed as an economical viable process for acquiring high grade vegetable oil [Galvão et al., 2013].

Microalgae is currently a hot topic due to several reasons. A few examples are that it is considered to be the third generation source for biofuels [Gouveia and Oliveira, 2009], it could be used in clean up processes of e.g., flue gases [Jin et al., 2005], it could be used for production of nutraceuticals [Herrero et al., 2006] and is also a food source [Draaisma et al., 2013]. The use of microalgae can be multi-purpose in order to strengthen the economics of using microalgae [López Barreiro et al., 2014]. Naturally, this has led to an extensive search for applications of SFE of various compounds from microalgae. For example, bioactive lipids [Andrich et al., 2005], carotenoids [Pan et al., 2012] and fat-soluble vitamins [Michalak et al., 2015] have been extracted from microalgae using SFE.

SFE also has a strong position within analytical chemistry [Švarc-Gajič, 2012]. Obviously, in any of the studies mentioned above analytical chemistry scaled SFE has been an essential tool in order to acquire chemical measurements. SFE in chemical analysis stretches much further though. Actually, SFE could essentially be the technique of preference in any case where hydrophobic compounds need to be extracted from a solid matrix. Hence, the technique is widely applied in the analysis of food, soil and plant material. For example SFE has been widely applied in the analysis of fat-soluble vitamins in food [Turner and Mathiasson, 2000], polycholorinated biphenyls in sediments [Nilsson et al., 2002] and pesticides residuals in crops [Ono et al., 2006]. These are mere examples, and the list of applications goes beyond what can be covered in this thesis.

In this thesis the fundamental phenomena in SFE applied to crushed

linseed and microalgae have been studied. It is enjoyable to see that the fundamental research is of interest on both large scales and small scales. An extended knowledge will help designing new applications and methods as well as improve already well-known applications.

Chapter 3

Experimental measurements

> *Without meaningful data on real mixtures, the set of modeling and design equations can only be solved for simple cases, which may render the solution irrelevant.*

Gerd Brunner, *Gas Extraction*, 1994

3.1 The study of extraction curves

The study of extraction kinetics has a long history and there has been numerous studies published over the years. A search using The Web of Science search engine with the key words ”supercritical fluid extraction” and ”kinetics”, generated 290 hits (performed in May 2016). Although the niche research area spans about 30 years of research, almost all information is derived out of extraction curves. For example already in 1990 Goto et al. [1990] studied the SFE kinetics of lignin from wood, using extraction curves. Yet today this is the most common methodology to obtain data for studying kinetics, simply due to the difficulty of performing any actual measurements of the sample matrix, continuously and inside the actual extraction vessel.

The extraction curves are almost exclusively generated by performing SFE and simply collecting fractions over time. The accumulated analyte concentrations of the fractions then generate the extraction curves, where the accumulative extracted amount is plotted typically versus time. However, collecting fractions during dynamic extractions is not a trivial task. The formed gas due to depressurization of the $scCO_2$ after the BPR makes the task difficult in practice. Volatile compounds may simply be lost along with the gas flow [Turner et al., 2002]. Particularly, the collection solvent

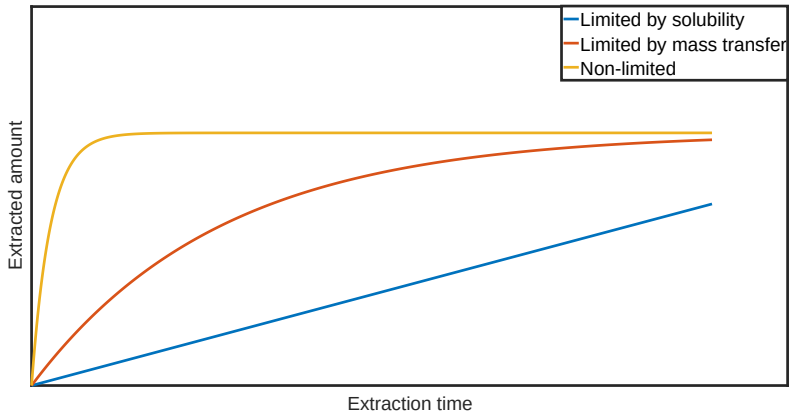

Figure 3.1: An illustrative example of the characteristic extraction curves, typically discussed in the literature [Clifford, 1999]. From top to bottom, an extraction curve which is not limited (yellow), an extraction curve limited by mass transfer (red) and an extraction curve limited by solubility (blue). The latter is especially noted as the accumulated extracted amount is linear, thus the extraction rate (the derivative) is constant. A combination of both types may very well characterize different stages of an extraction. Note that although the profile usually corresponds to the type of limitation, there is no guarantee that so is the case [Brunner, 1994].

upon using capillary restriction has been suggested to influence the recovery of analytes [Hedrick et al., 1992]. Systematic errors like these can easily bias any extraction curve if proper care is not taken. As with any systematic errors, these are not easily found [Miller and Miller, 2010]. The fraction collection also implies daunting laborious work, rendering the experiments expensive in terms of manpower. Most likely, this is why relatively few experiments are performed in general associated with previously published work found in the literature.

The acquired extraction curves can subsequently be inspected visually (Figure 3.1). The curves can in a very simplified manner be divided into two parts, namely, solubility and kinetic limitations of the extraction rates [Švarc-Gajič, 2012]. The linear part of the extraction curve consequently represents the solubility limited stage of the extraction process, while the part of the diminishing extraction rate represents the kinetic limitation. These general evaluations are frequently applied to SFE curves as well [McDaniel et al., 1998, Sovová, 1994].

The acquired extraction curves can be of later use in order to perform inverse modeling to gain further insights into the underlying phenomena of the SFE. This is, however, thoroughly covered in the Chapter 5.

3.2 Channeling effects

In the previous chapter where the principles of SFE were discussed, channeling effects were briefly mentioned. It was stated that channeling effects arise due to difference in flow velocity profiles through a packed bed facilitated by heterogeneous hydraulic resistance [Sovová, 2006]. In other words, the fluid has a tendency of mainly flowing through void volumes rather than spaces inside the extraction vessels which are tightly packed (Figure 3.2). This issue has been a consistent concern throughout this work, and might also stir some controversy. Therefore, a whole section within this chapter is dedicated to channeling effects explicitly.

Channeling effects, i.e., lack of contact between the bulk fluid and parts of the stationary bed, can cause lower recoveries as the extraction converges towards a complete extraction. From an analytical chemistry point of view this results in a systematic error, which will bias and result in a lower reported value in comparison to the true concentration of an analyte in a solid sample. Independently if the purpose is to perform conventional quantification or to study extraction curves, systematic errors like these either need to be eliminated or completely accounted for.

It is particularly interesting that very few studies, or at least, no dedicated study within the research field of SFE has given attention to channeling effects. Still, for example in many studies glass beads or sand is added with the motivation that it acts as a dispersion agent and limits channeling effects [Chun et al., 1996, Lang and Wai, 2001, MacHmudah et al., 2012, Safaralie et al., 2008, Yamini et al., 2002]. In some instances the glass beads are mixed with the sample, in other cases it is added at the top and bottom of the extraction vessel. Based on an overall evaluation of the available literature, there seems to be little consensus on the packing configuration, although an agreement that they are efficient at eliminating channeling effects is apparent. However, it is important that the effects of adding glass beads are seldom evaluated. Recovery studies of spiked matrices are unfortunately not a general practice. These might indicate if channeling effects are present, however, may not necessarily be a reliable method for correctly estimating the extraction recovery of a method. It has been shown that spiking has an age effect, namely spiking over a longer period of time renders a larger faction of analytes unextractable [Björklund et al., 1999].

Besides the inconclusiveness regarding packing configuration, it is quite clear that a high moisture content may cause agglomeration, consequently resulting in channeling effects [Del Valle et al., 2012].

The presence of channeling effects was observed upon performing ex-

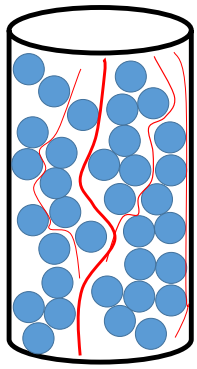

Figure 3.2: A simple schematic of a plausible channeling effect scenario. The particles are not uniformly packed and a larger fraction of the solvent is flowing through formed channels in the packed bed. Some fractions of the solid material may not be in contact with the extraction solvent.

traction from both crushed linseed (**Papers II-III**) and microalgae (**Paper IV**). Additional extractograms which were not presented in **Paper II** are shown here (Figures 3.3-3.4). In **Paper I**, carotenoids were extracted by SFE, however, the main purpose was to develop a chromatographic method for the separation of the extracted carotenoids rather than developing a full analysis method. Thus, proper evaluations of the extraction method were not performed.

Two very different principle routes were taken in order to deal with the channeling effects in the two cases of the linseed and microalgae. Each of the two approaches described below has its own advantages and disadvantages in regards to both quantitative analysis and how to approach the inverse modeling. The issues of the latter are discussed in Chapter 5.

The presence of channeling effects can be tested by performing additional re-extractions. This is done by rapid depressurization of the extraction vessel, and potentially by also manually re-mixing the sample in between extractions. This was the general approach in this work, and is neatly described and visualized in **Paper II**. Other authors have reported rupturing of the sample matrix, e.g., cell walls. However, the channeling effects and reported rupturing of the sample matrix, is most likely not distinguishable through this methodology. For example, Barthet and Daun [2002] reported that multiple extractions of various oil seeds increased the recovery of oil. The same authors claimed that this phenomenon was due to the sample

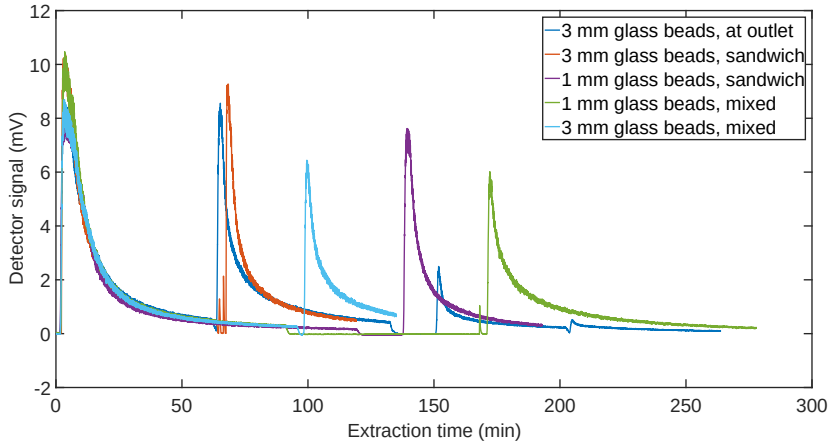

Figure 3.3: Studies of various packing configurations and their influence on channeling effects during SFE applied to crushed linseed. The same extraction conditions and sample amount was used in all experiments. The detector response is proportional to the total lipid concentration in eluent. An initial extraction was performed. After at least 50 min of extraction, a depressurization of the vessel was performed and another extraction was started. The figure shows that channeling effects occur independently of whether 1 mm or 3 mm diameter glass beads, packing the sample between the layers of glass beads (sandwich), mixing it, or by just filling the void volume at the end of the extraction vessel. This is characterized by the analyte concentration in the eluent converges towards zero and that after a depressurization step more analytes can be extracted.

matrix opening up between each pressurization and depressurization step. Another example is Sahena et al. [2010] who referred to this procedure as pressure swings, where the authors claimed that the sample matrix was ruptured by these.

There are many claims in the literature that rapid depressurization ruptures e.g., oil seeds. However, Fattori et al. [1988] compared various pretreatments of canola seeds prior to SFE. Although the authors reported that virtually no oil was extracted without any pretreatment, rapid depressurization had almost no impact in comparison to crushing, chopping or flaking and cooking.

In summary, there seem to be consensus in the literature that rapid depressurization causes rupturing of the sample matrix and thereby increasing the amount of analyte accessible for the extraction solvent. The reported results are, however, conflicting. No previous work has reported that a rapid depressurization might cause a reconfiguration of the packed bed inside the extraction vessel. In **Paper II**, it is shown that using a fully packed and a smaller extraction vessel with several depressurization steps results in the

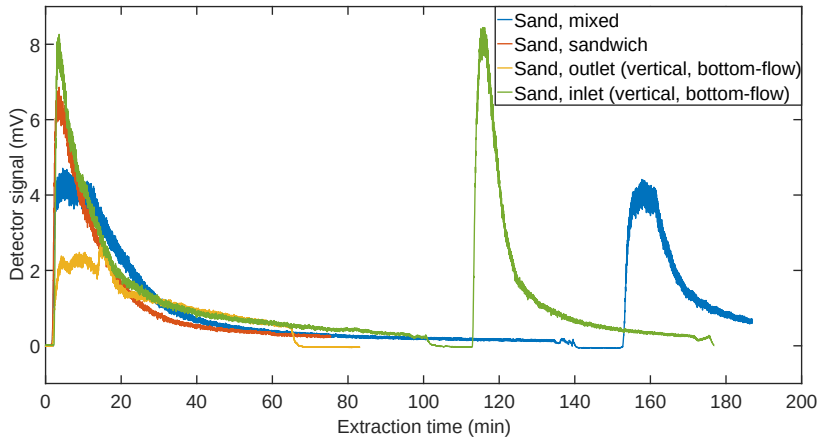

Figure 3.4: Studies of channeling effects by various configurations of added sand as a dispersant agent during SFE of lipids from crushed linseed. The same extraction conditions and sample amount was used in all experiments. Measurements were performed using ELSD.

same accumulated yield as by one single extraction cycle using the same sample amount in a severely oversized extraction vessel. These findings suggest that the increased yield reported in previous works is due to a reconfiguration of the packed bed, thus forming new flow paths through the bed and therefore counteracting present channeling effects.

In the case study where lipids were extracted from crushed linseed, it was concluded that experiments were repeatable (**Paper II**). The channeling was taken into account during the inverse modeling and no experimental alterations were performed (**Paper III**). However, in the other case study of the microalgae, the experiments were not repeatable (**Paper IV**). An alternative approach was employed to circumvent the channeling effects by only filling the extraction vessel by one third and by positioning it vertically with the inflow of extraction solvent from the bottom. This enables natural convection and turbulent flow inside the extraction vessel (Figure 3.5). However, it was also noted that higher flow rates, i.e., above 1.0 mL/min, were needed in order to reach a complete extraction. Lower flow rates did not induce enough stirring to ensure that all of the sample came in contact with the extraction solvent. This of course affects the approach of the inverse modeling and the design of experiments, which is discussed further in Chapters 5-6.

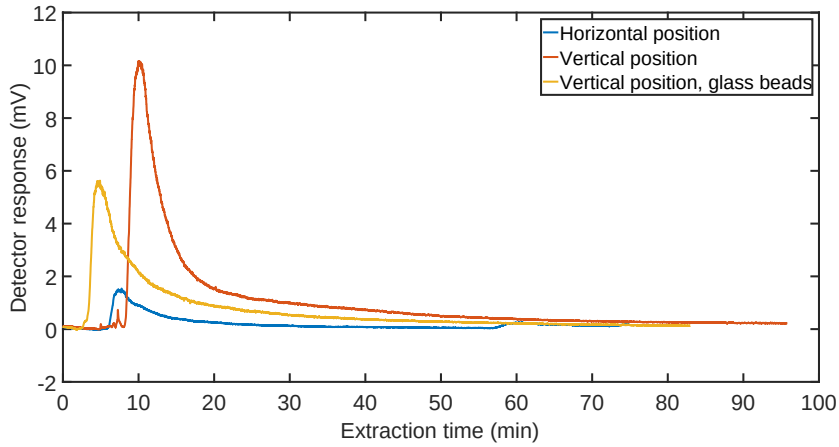

Figure 3.5: Examples of extractograms which show the influence of the extraction vessel positioning when extracting from microalgae *Chlorella* sp. Detection was performed using on-line ELSD. Extractions were performed at 30 MPa and 40 °C with neat CO_2. The blue line shows the extraction rate when the extraction vessel was positioning horizontally at a flow rate of 0.5 mL/min. By positioning the extraction vessel vertically with the inlet from the bottom (1.5 mL/min) a much higher yield was acquired (red line). By adding glass beads (yellow line), the extraction yield was diminished even though the extraction vessel was positioned vertically at the same conditions. The glass beads hinder the stirring of the sample.

3.3 Instability of analytes

Another critical aspect of performing chemical analysis is the consideration of unstable compounds. In **Papers I, IV-V** carotenoids, chlorophyll A and ergosterol were extracted from microalgae. All of the compounds are of general interest due to their antioxidative capacity, which could be of interest as nutraceutical or as food additivies [Herrero et al., 2013, Rao and Rao, 2007, Sahena et al., 2009]. This property is related to the system of conjugated double bonds, which gives rise to easily oxidized compounds [Boon et al., 2010]. Consequently, certain precautions are needed in order to minimize the influence of degradation on the final results. This is discussed further down. Degradation can never be entirely avoided, however, it does contribute towards a bias of the chemical analysis.

Carotenoids are particularly sensitive, especially towards oxygen exposure but degradation is also facilitated by light, heat and acidity [Boon et al., 2010]. Standards are difficult to acquire at a high purity and at a reasonable price. These are frequently acquired in a prepared solution that is encapsulated in an oxygen-free environment shielded from light. However, some

standards can be bought in the dry form. It is important to highlight that the typical procedure for preparing these standards are to simply determine the concentration of the stock solution by means of spectrophotometry [Thomas et al., 2012]. The determination, or rather estimation, is based on reported molar absorption coefficients of isolated compounds. Consequently, the path of traceable uncertainty becomes slightly unconventional. Throughout the years, numerous coefficients have been determined for the various carotenoids, all with slightly different values. The common pragmatic approach is to utilize the highest reported molar absorption coefficient [Roy, 2011]. This introduces an unknown bias towards any future estimation of samples which cannot be corrected for.

It is safe to state that analysis of photosynthetic pigments presents a very difficult challenge. The before-mentioned systematic errors can, and of course should, be minimized by storing samples and standards at low temperature, free from oxygen, light and low pH. This is achieved by preferably storing standards and stock solutions at -80 $^{\circ}C$, in amber vials and covered by aluminium foil and by purging the solution and filling the head space with nitrogen or argon. The concentrations of the prepared stock solutions should be tested at least weekly if routine analyses are performed. Naturally, the stability and degradation rate depends highly on the compound, but a conservative approach should be applied as it is better to be safe than sorry.

3.4 On-line measurements

At this point it is clear that collection during SFE is problematic, and is further reinforced upon dealing with unstable analytes such as e.g., carotenoids. An alternative approach is to utilize in-line or on-line detection methods in combination with SFE [Amador-Hernández and Luque De Castro, 2000]. Examples of various categories of experimental measurements are presented in Figure 3.6. For example, several authors have reported the use of hyphenated chemical detectors primarily using spectrophotometry in the infrared region [Heglund et al., 1994, Liang and Tilotta, 2003, Ramsey, 2008], but also in UV spectral region [Goto et al., 1990]. Amador-Hernández and Luque De Castro [2000], although slightly out-dated, has summarized previous works regarding the hyphenation of SFE and chemical detectors.

Hyphenation with chromatographic systems is also an option, which has been quite thoroughly investigated. If the purpose is to acquire extraction curves, it is necessary to consider the sampling rate during the SFE process. The sampling rate will in this case correspond to the total chromatographic

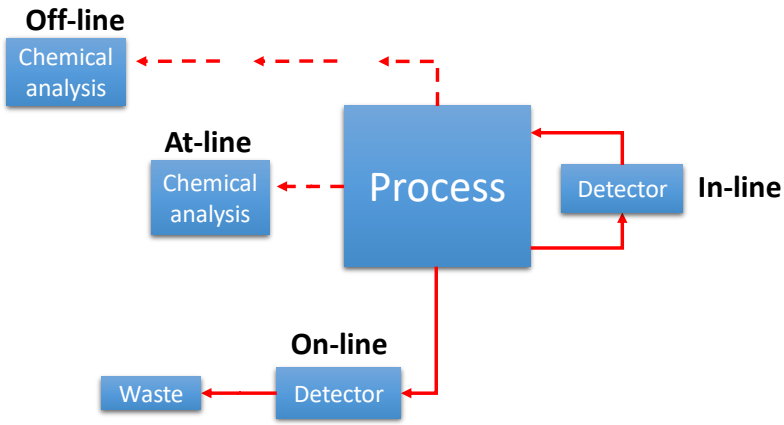

Figure 3.6: Various ways of performing measurements according to Hassell and Bowman [1998]. The definitions are not clear-cut. Off-line analyses are performed out of vicinity of the process. At-line analysis means that measurements are performed close to the process. Both of these require manual sampling. On-line measurements involve automatic sampling and transportation to the analyzer. In-line measurements are performed in the process itself.

analysis time of the hyphenated chromatography system. It is difficult to judge prior to any investigation how many sampling points are needed. If an hour is needed in order to reach a complete extraction, a total chromatographic analysis time of ten minutes will only result in six sampling points. Figure 3.7 illustrates a scenario in which a high sampling rate is required.

Furthermore, incorporating a chromatography system means that additional units such as pumps and valves are needed. Every additional component in a system is an added risk of instrumental failure. Nonetheless, if the extracted mixture of analytes is complex, on-line or off-line chromatographic analysis may be the last resort. The interested reader is recommended to read the works of Pól et al. [2004], Wang et al. [2003], Wang et al. [2004] and Wang et al. [2005].

As will become apparent in Chapter 5, many experiments are needed in order to perform meaningful inverse modeling. The labour intensity of manually collecting fractions during the SFE process along with the following off-line analysis has been one of the main driving forces in the development of in-line and on-line detection in combination with SFE applied in this work.

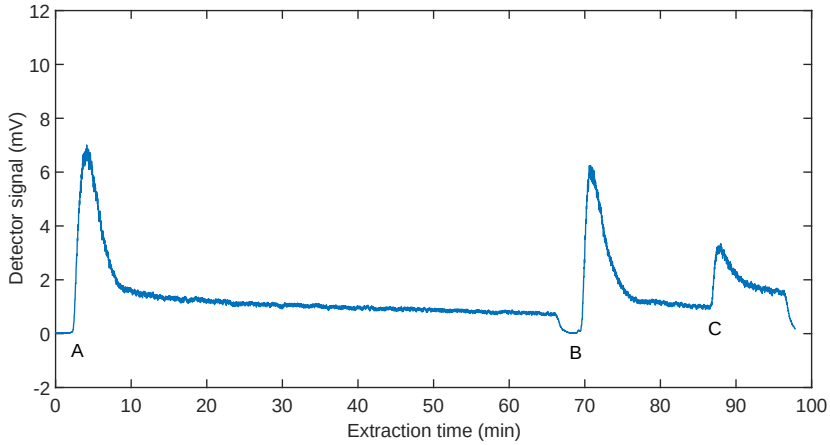

Figure 3.7: Illustration of phenomena which could most likely only be studied by on-line detection (e.g., ELSD) with high sampling frequency. Lipids were extracted from crushed linseed mixed with 5 mm diameter glass beads. The extraction was initialized (A), the extraction vessel was depressurized and then the extraction was continued (B), the extraction vessel was then swiftly hit by a wrench and thereby causing a stirring effect inside the extraction vessel (C).

In **Paper II**, a methodology based on SFE in combination with ELSD was proposed. The ELSD is an almost universal detector which is very suitable for compounds which do not sufficiently absorb light in the UV/Vis spectral region [Poole, 2003]. It is a common detector for both liquid chromatography (LC) and SFC analysis. As the dissolved analytes enter the ELSD they are nebulized, then the solvent constituting the droplets is evaporated in a drift tube and dry particles are formed. A light beam is positioned at the end of the drift tube where the solid particles scatter the light. The scatted light is measured by a photosensor positioned with an angle to the light beam. Hence, volatile compounds which do not form particles will not be detected.

As the overall process of the ELSD involves nebulization, which is usually facilitated by an external source of nitrogen, the feed of CO_2 from the SFE is not an issue. The detector response is usually only perceived as linear in a rather narrow range of analyte concentrations [Poole, 2003]. Nonetheless, the ELSD is usually equipped with a gain factor setting enabling operations at a wider range of concentrations. Lesellier et al. [2012] performed an extensive investigation on which parameters affect the response of the ELSD when hyphenated with SFC. Among several interesting findings, the authors concluded that flow rate and mobile phase composition affected the

response factor and that the flow rate also affected the nebulization chamber temperature, which in turn also affect the response factor.

The findings of Lesellier et al. [2012] have some practical implications which were also observed in **Paper II**. For example, it was observed that sufficient time of pumping solvents was needed for the detector to reach a stable response factor. It is then important that the solvent feed composition and flow rate to the ELSD is the same during calibration, pre-extraction and during the actual extraction. Adjustments of the post-BPR heating will also affect the temperature of the nebulization chamber. Approximately half an hour was sufficient in the beginning of the day, in order to reach steady-state temperatures of the whole setup.

Yet another consideration arising from the combination of using a BPR and an ELSD is the transport of extracted analytes between the two units. As the $scCO_2$ expands into a gas the solubility of analytes drops severely. To ensure a complete and steady flux of analytes a make-up flow of ethanol was introduced. In **Paper II-III** a make-up flow corresponding to the equal amount of pumped liquid CO_2 was needed at a post-BPR temperature of 90 $^{\circ}C$. However, it was later discovered that lower make-up flows were needed when a lower post-BPR temperature was employed (**Paper IV-V**). At 40 $^{\circ}C$ the make-up flow only needed to correspond to one third of the pumped CO_2. The most likely explanation is that the ethanol used as a make-up solvent is at least partially evaporated, thereby diminishing the solubility. The option to use a lower fraction of alcohol is very convenient, since this increased the lifetime of the seals in the BPR, as was mentioned in Chapter 2.

In **Papers IV-V** carotenoids, chlorophyll A, ergosterol and total lipids were extracted and therefore additional detector selectivity was needed. The instrumentation was thus further developed in **Paper IV** by adding in-line spectrophotometric detection. This allowed for improvements in detection selectivity and consequently phytopigments from microlagae could be determined (**Papers IV-V**). The implementation is very straight-forward by simply incorporating a flow cell with optical connections to a charged-coupled device (CCD). Unfortunately, at the moment when this thesis is being written, relatively few options of flow cells are available. In **Papers IV-V** a 1 cm optical path length was utilized, however, in future applications a smaller size flow cell is recommended as the molar absorption coefficients of phytopigments are in general very high [Roy, 2011]. The proposed setup has an additional CO_2 pump, enabling dilution of the extraction solvent prior to flowing through the flow cell. Adjustment of the sample amount was also needed in order to keep within the linear range of the spectropho-

tometer.

The raw data acquired from the spectrophotometer is of little use if analytes with overlapping absorption spectra are co-extracted. Necessary signal processing and curve resolution techniques are discussed in Chapter 4.

The last matter at hand is the calibration of the detectors if the purpose is to do quantitative analysis rather than qualitatively evaluate the extraction curves. Previously published works have mainly focused on evaluating the feasibility of on-line detection. The proposed instrumental setup of **Paper II** and **Paper IV** incorporates a HPLC injection valve with a fixed size loop of a known volume. The calibration of the ELSD is straight-forward as calibration solutions are injected through the injection valve. The calibration of the spectrophotometer is slightly more cumbersome, due to baseline shifts which occurs probably due to the pressure impulse, i.e., change in density, and the different composition of the injection plug. This issue is addressed by some signal processing, described in Chapter 4.

An alternative feasible approach for spectrophotometric measurements, which has not been evaluated in this work, is to utilize reported molar absorption coefficients of the known analytes. If the temperature of the extraction solvent is known in the flow cell, then both the concentration and the flow rate could be estimated and thereby also the total amount of extracted analyte.

3.5 Off-line analysis using supercritical fluid chromatography

In some scenarios off-line, or at-line, analysis is indispensable. For example, if the on-line detectors are not selective enough for target compounds. In **Paper IV** the total amount of extracted carotenoids was quantified, however, very scarce information is given in regards to the overall composition of carotenoids. Other types of off-line analysis might be needed for confirmatory purposes and validation.

Chromatographic analysis of carotenoids is a particularly interesting challenge. The actual interest of carotenoids in economically feasible applications have already been mentioned in Chapter 2. Traditional methods resort to LC, most typically using either C_{18} or C_{30} columns and exclusively employing an organic mobile phase [Oliver and Palou, 2000, Sander et al., 2000]. Thus primarily focusing on hydrophobic interactions between the stationary phase and the analytes, while the sterical hinderence play a key role when using a C_{30} column [Lippa et al., 2005]. Many of the present

carotenoids in microalgae are indeed extremely similar [Koller et al., 2014]. As is the case comparing lutein and zeaxanthin, where a shift of double bond is what distinguishes them apart (structures are visualized in **Paper I**).

The traditional methods therefore involve plenty of rather unpleasant organic solvents such as dichloromethane, hexane or methyl tert-butyl ether which are highly flammable and toxic. Furthermore, the total analysis time is relatively long, depending on the complexity of the sample [Meléndez-Martínez et al., 2007, Oliver and Palou, 2000]. It is not uncommon with LC methods exceeding one hour of total analysis time.

Therefore, a method based on SFC was developed, where only 10 minutes were needed in order to separate 8 carotenoids common in microalgae (**Paper I**). The method was based on coupling a C_{18} in-front of a 2-ethyl pyridine (2-EP) column and using a diode array detector (DAD) for detection. The method which utilizes only CO_2 and methanol as mobile phase, and due to the short analysis time can be considered more environmental benign in terms of solvent usage.

Since then plenty of alternative stationary phases have become easily available through commercial companies. That in combination with the rapid development of SFC over the last few years have resulted in even more improved methods. For example, Jumaah et al. [2016] developed a method based on ultra high-performance supercritical fluid chromatography (UHPSFC) and a 1-aminoanthracene column, where a similar mixture of 10 carotenoids were separated in less than 6 minutes.

Chapter 4

Data processing

> *Measure what is measurable, and make measurable what is not so.*

Galileo

4.1 Data management

Up until this point, much of the focus has been directed towards practical issues in the actual laboratory. Before dealing with issues regarding data treatment, a good structure of storing data is essential and will facilitate any subsequent batch manipulations or calculations.

All of the work in this thesis in regards to data handling, signal processing and so on has been performed using MATLAB (MathWorks Inc., Natick, MA). It does not imply that other platforms are not suitable. However, it is readily available at most universities, the language is relatively user friendly, the software already holds a large library of essential functions and open-source chemometric algorithms are published online. The platform was also used for inverse modeling (Chapter 5), which enabled an integrated approach.

In this work exported output from the ELSD and the spectrophotometer were saved as separate comma-separated values (csv) files along with an additional csv file containing information about the experiment (Figure 4.1). Other export formats are available and compatible with MATLAB, e.g., common data format (CDF) files. However, currently not all softwares have the functionality to export the acquired data into a raw format.

The exported raw detector data is a one dimensional vector for the ELSD and a two-way data array for the spectrophotometric data as full spectra

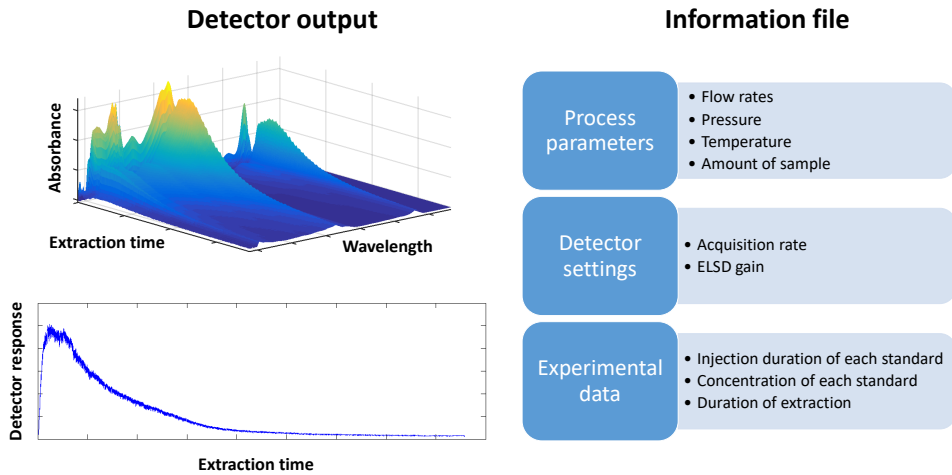

Figure 4.1: Overview of essential information and raw data derived and saved from each experiment. The detector output from the spectrophotometer (top) and the ELSD (bottom) are stored as csv files. Experimental conditions and other experimentally related information are manually entered into a csv file.

are stored during each measurement. A vector with the time points of the measurement is also accompanying the raw detector signal.

The information file contains information about process parameters, detector settings and the time duration of the response due to injected standards and so on. By structuring the data, the import of the information into MATLAB can be completely automated and the information which is needed during both curve resolution, calibration and during the inverse modeling becomes easily accessible. It is therefore highly recommended to decide on data formats and data structures prior to developing any further tools.

During import of the data it is important to utilize routines which perform signal processing (described in the next section) and data reduction of one experiment at the time. Because, if plenty of experiments have been performed, the amount of raw data may be larger than the available memory of the computer. Finally, in this work, the data was stored in a cell-array structure enabling easy access of data and experimental information (Figure 4.2).

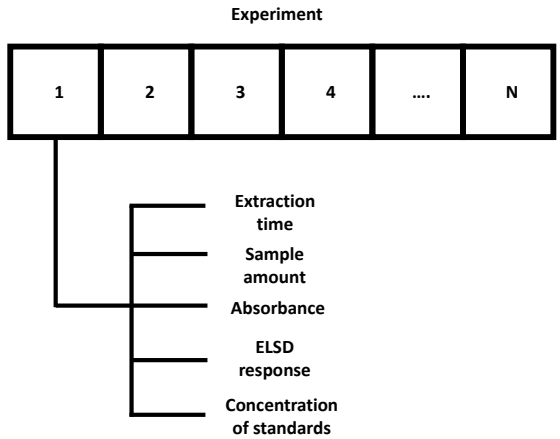

Figure 4.2: The data of each experiment is stored in a cell array. Each cell contains a structure array of e.g. measurements, extraction conditions and other relevant information. The figure is just a representation and in reality each cell contains all relevant information for signal processing and inverse modeling. This way of storing data enables swift removal and addition of experiments.

4.2 Signal processesing

The acquired raw data of the ELSD and the spectrophotometer can at times be relatively noisy. In the later part of this work, a Savitzky-Golay filter was applied in order to smooth the data prior to reducing the number of data points in the temporal dimension [Savitzky and Golay, 1964]. The algorithm performs an estimation of each measurement point based on the adjacent values using a polynomial function. A window size and the degree of the polynomial needs to be user specified. This filter was applied in **Papers IV-V**, first in the time dimension and then secondly in the spectral dimension of the spectrophotometric data. The window size and the polynomial degree was chosen based on visual evaluation the processed detector signals, as suggested by Rinnan et al. [2009].

During the injection of calibration standards, an intensity shift was observed over the entire spectra. This shift was corrected by utilizing a reference wavelength at 880 nm. The entire spectra were subtracted using the obtained absorption value.

The effects of preprocessing are visualized below. Figure 4.3 shows the spectral profiles of an injection of β-carotene standard before and after preprocessing using a reference wavelength and Savitzky-Golay filter. The smoothing of the elution profile during an extraction is visualized in

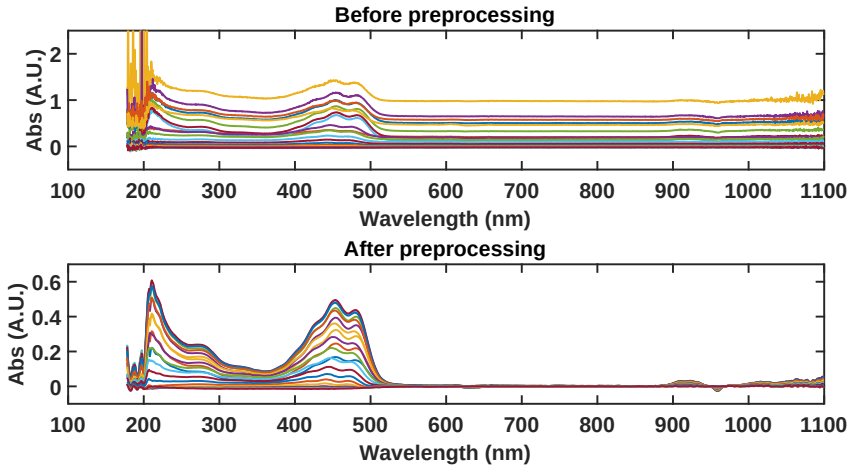

Figure 4.3: Overlaid spectra acquired during an injection of β-carotene. A baseline shift is observed during the injection which is corrected by using a reference wavelength at 880 nm.

Figure 4.4.

Finally, the data was reduced by only retaining the essential number of measurements during the extraction period prior to storing the processed data. No data reduction was performed of the measurements acquired during calibration in order to maintain a reasonable number of measurements over the duration of the injection.

4.3 Curve resolution

Curve resolution techniques refer to mathematical ways of resolving pure analyte spectra and analyte concentration profiles from data on chemical mixtures, which is usually obtained from multi-channel detectors. The most commonly used multi-channel detectors used in analytical chemistry are the CCD and the DAD, which are spectrophotometers, and mass spectrometry (MS). Particularly, curve resolution of chromatographic data has been of interest in previously published work [Amigo et al., 2008, 2010, Bordagaray and Amigo, 2015, Johnsen et al., 2014].

In this work only spectrophotometric measurements have been acquired for the purpose of in-line measurements. This is a very popular approach within the field of process analytical technology (PAT) and is particularly applied in the pharmaceutical and the food industry [Luypaert et al., 2007, Swarbrick, 2014, Tajammal Munir et al., 2015].

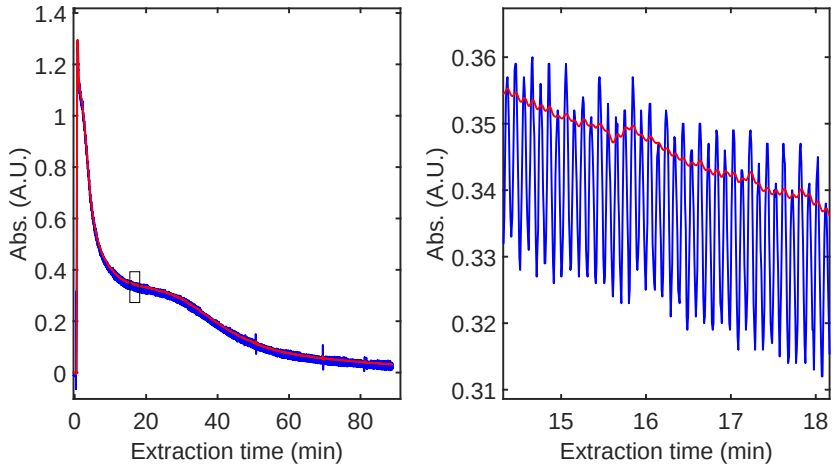

Figure 4.4: Example of the acquired spectrophotometric data at 451 nm during a SFE experiment with microalgae. The blue lines represent the raw data and the red line shows the preprocessed data. The right figure shows a zoomed part of the extraction curve.

One of the main assumptions is that the data is at least billinear by following the linear model of Lambert-Beers law at all of the wavelengths:

$$A = c \times \ l \times \ \epsilon \tag{4.1}$$

where A is the absorbance, c is the concentration, l is the optical path length and ϵ is the molar absorption coefficient.[1]

Another necessary requirement is that the data is of appropriate pseudo-rank. From an applied setting, rank refers to the information content of a matrix. The definition of rank is maximum number of linearly independent row vector or column vectors in a matrix. A linear system which is not of full rank cannot be solved. The usual mathematical definition is of little use when performing modeling of experimentally acquired data. This is due to random measurement errors introduced by the chemical analysis. For example, a matrix of any size with perfectly random numbers will have an extremely low probability of being rank-deficient. Therefore, if 10 solutions of only one analyte at various concentrations are measured by spectrophotometry at a range of wavelengths, the rank should be of one. However, due

[1]The same variable notation is used as by Smilde et al. [2004]. Scalars (x) are denoted by lower-case letters and vectors ($\mathbf{x}$) are denoted as bold-faced lower-case letters. Bold-faced capitalized variables denotes two-way arrays ($\mathbf{X}$), i.e., a matrix. Underlined, bold-faced capitalized variables denote three-way arrays ($\underline{\mathbf{X}}$), i.e., a tensor.

to measurement precision, the obtained data matrix will most certainly be of mathematical rank 10.

Instead, pseudo-rank or chemical rank are more useful term in this context [Smilde et al., 2004]. In the above example, the pseudo-rank would be one. The pseudo-rank will always be lower than the mathematical rank. Still, the pseudo-rank can still be lower than the chemical rank. This will occur when the concentration of two or more analytes covary or when the pure spectra are identical [Smilde et al., 2004]. For example, in **Paper IV**, the different carotenoids have both similar spectra and are most likely extracted at a very similar rate. Therefore, these cannot be distinguished.

This is one of the main limitations of the current curve resolution techniques. It is required that, at least in some measurements, all analytes vary independently and that the spectra are not too similar. Otherwise the pseudo-rank will be lower than the chemical rank [Smilde et al., 2004]. It should be mentioned that methods do exist to handle some of these issues, for example, using parallel factor analysis with linear dependence [Bordagaray and Amigo, 2015].

It is of the essence that the data is of low rank, but preferably not of lower pseudo-rank than chemical rank. This could be the case if the extraction rate of two or more analytes are very similar for all performed SFE experiments.

It is important to have a basic knowledge of the mathematical framework of the various techniques and the used numerical methods. These help to understand the properties, abilities and limitations of a technique like parallel factor analysis (PARAFAC). This is gained through experience and is very much facilitated by a understanding the basics of what the algorithms are calculating. For example, an unrealistic model might be acquired because the numerical method failed. Some of the relevant basics are presented below.

4.3.1 Classical least squares

Classical least squares (CLS) is not actually a curve resolution technique, however, the principles remain the same. If several multi-wavelength spectrophotometric measurements are performed, the data is a linear combination of the absorption spectra and the concentrations (Eq. 4.2).

$$\mathbf{X} = \mathbf{C}\mathbf{S}^T + \mathbf{E} \tag{4.2}$$

If the pure spectra, $\mathbf{S}$, of each chemical compound is known, and they are unique, the concentration, $\mathbf{C}$, in a mixture can be estimated from the data spectrophotometric data $\mathbf{X}$. The random measurement error is then account

for by $\mathbf{E}$. [Brereton, 2006]. The dimensions of the matrices are $\mathbf{X}(I \times K)$, $\mathbf{C}(I \times N)$, $\mathbf{S}(J \times N)$ and $\mathbf{E}(I \times J)$, where I is the number spectra, also corresponding to time if the measurements frequency is constant, J is the number of spectrophotometric channels measured and N is the number of chosen components for the model.

The estimation of the concentration $\hat{\mathbf{C}}$ is performed according to Eq. (4.3).

$$\hat{\mathbf{C}} = (\mathbf{S}^T\mathbf{S})^{-1}\mathbf{S}^T\mathbf{X} \tag{4.3}$$

The above expression, $(\mathbf{S}^T\mathbf{S})^{-1}\mathbf{S}^T$ is also known as the pseudo-inverse, sometimes denoted $\mathbf{S}^+$, and is commonly applied in least-squares minimizations. Proofs, theory and further applications can be found in any standard text book on linear algebra, for example by Lay [2012].

4.3.2 Multivariate curve resolution

If neither the concentration nor the spectra of each compound is known, then CLS is not a viable option anymore. Fortunately, multivariate curve resolution alternating least squares (MCR-ALS) offers an approach to simultaneously estimate both the concentrations, $\hat{\mathbf{C}}$ and the pure spectra, $\hat{\mathbf{S}}$.

The most popular algorithm is the one propsed by Tauler [1995], where the model is fitted using a singular value decomposition (SVD), i.e., principal component analysis (PCA), decomposed data matrix, $\hat{\mathbf{X}}_{PCA}$, using the same number of components as for the multivariate curve resolution (MCR) model [Ruckebusch and Blanchet, 2013]. The unknowns, $\hat{\mathbf{C}}$ and $\hat{\mathbf{S}}$ are re-estimated by alternatively minimizing the objective functions according to Eqs. (4.4)-(4.5) [Tauler, 1995].

$$\min_{\hat{\mathbf{C}}} \|\hat{\mathbf{X}}_{PCA} - \hat{\mathbf{C}}\hat{\mathbf{S}}^T\| \tag{4.4}$$

$$\min_{\hat{\mathbf{S}}^{\mathbf{T}}} \|\hat{\mathbf{X}}_{PCA} - \hat{\mathbf{C}}\hat{\mathbf{S}}^T\| \tag{4.5}$$

This is typically done using the pseudo-inverse as described in Eq. (4.3). However, this is an ill-posed problem as no unique solution exists. Even in a system with a full pseudo-rank, rotational ambiguities are present:

$$\mathbf{X} = \hat{\mathbf{C}}\hat{\mathbf{S}}^T = \hat{\mathbf{C}}\mathbf{R}^{-1}\mathbf{R}\hat{\mathbf{S}}^T \tag{4.6}$$

where $\mathbf{R}$ is a rotation matrix.

By imposing either hard or soft constraints on Eqs (4.4)-(4.5), a desired solution can be obtained. In this work non-negativity constraints on both $\hat{\mathbf{C}}$ and $\hat{\mathbf{S}}$ were applied. This is simply motivated by that no negative concentration nor absorption should be physically possible.

4.3.3 Parallel factor analysis

The benefit of progressing from multivariate to multiway techniques is that it allows for more parsimonious models [Smilde et al., 2004]. Models become easier to interpret as data can be retained in its original form without needing to unfold it into a matrix. An illustration of unfolding of a three-way array into a matrix is given in Figure 4.5.

It was shown before that prior to MCR the sample mode was unfolded, into what could be seen as one long run of several experiments. PARAFAC maintains the sample mode and thus decomposes the data cube $\underline{\mathbf{X}}$ into three loadings corresponding to relative concentrations (sometimes also called scores), extraction profiles and the absorption spectra (Eq. (4.7)).

$$\mathbf{X}_k = \mathbf{A}\mathbf{D}_k\mathbf{B}^T + \mathbf{E}_k, k = 1, ..., K \tag{4.7}$$

The notation is represented by $\mathbf{A}$, corresponding to the pure spectra and is also the same as $\mathbf{S}$ used in the MCR notation. $\mathbf{D}_k$ holds the relative concentration of each component in its diagonal of the k:th sample, $\mathbf{B}$ corresponds to the extraction profile and $\mathbf{E}_k$ is the residuals for the k:th sample. The PARAFAC model can also be written in summation notation instead:

$$x_{ijk} = \sum_{r=1}^{R} a_{ir}b_{jr}c_{kr} + e_{ijk}, i = 1, ..., I, j = 1, ..., J, k = 1, ..., K \tag{4.8}$$

were R is the number of components used in the model.

PARAFAC, however, require trilinearity which in practice means that the extraction profile needs to be identical throughout all experiments if the number of components should equal the number of modeled compounds [Amigo et al., 2008].

The extraction profiles are, however, not identical throughout all experiments. In fact, the experiments should most likely be designed to acquire as different extraction profiles as possible, as is described in Chapter 6. Parallel factor analysis 2 (PARAFAC2) is an extension of PARAFAC which allows for some shifts in one mode [Kiers et al., 1999]. In this particular case, the flexible mode is the one representing the extraction profile $\mathbf{B}$. This is done by estimating a unique extraction profile for every slab of $\underline{\mathbf{X}}$, i.e. for every experiment (Eq. 4.9).

$$\mathbf{X}_k = \mathbf{A}\mathbf{D}_k\mathbf{B}_k^T + \mathbf{E}_k, k = 1, ..., K \tag{4.9}$$

In order for Eq. (4.9) to be unique, additional constraints are needed [Kiers et al., 1999]. These are given by Eqs. (4.10)-(4.12) and renders $\mathbf{B}_k^T\mathbf{B}_k$ constant [Ten Berge and Kiers, 1996].

$$\mathbf{B}_k = \mathbf{P}_k\mathbf{H}, k = 1, ..., K \tag{4.10}$$

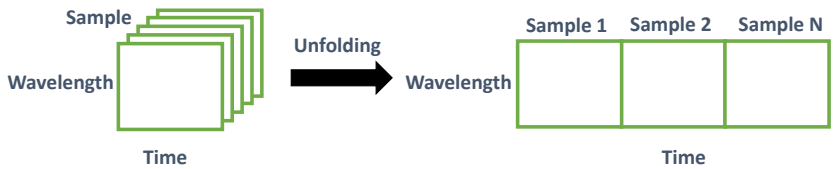

Figure 4.5: A schematic view of unfolding of a three-way data array into a matrix.

$$\mathbf{P}_k^T \mathbf{P}_k = \mathbf{I}, k = 1, ..., K \tag{4.11}$$

$$\mathbf{B}_k^T \mathbf{B}_k = \mathbf{H}^T \mathbf{B}_k \mathbf{B}_k^T \mathbf{H} = \mathbf{H}^T \mathbf{H}, k = 1, ..., K \tag{4.12}$$

The newly introduced variables **P** and **H** handle what is unique and common among the samples, respectively.

It is important to highlight that PARAFAC2 only allows for some shifts, differences or distortion of the extraction profile according to this estimation method [Kamstrup-Nielsen et al., 2013]. These mentioned properties of PARAFAC2 can be exploited in order to evaluate the appropriateness of a calibrated model, by using so called core consistency diagnostics [Kamstrup-Nielsen et al., 2013]. After a PARAFAC2 model is acquired, it can be rewritten so that a PARAFAC model can be fitted according to traditional core consistency diagnostics. This tool will in most cases indicate whether a model is unique, i.e., successfully able to decompose the data matrix. More in-depth theory is given by Bro and Kiers [2003] and Kamstrup-Nielsen et al. [2013].

This is particularly useful, because it is an everlasting issue to determine how many components a model should contain. In PARAFAC this is done by manually evaluating residuals, fit, loading profiles and through core consistency.

In **Paper IV**, it was shown that PARAFAC2 did not perform as well as MCR in the specific application. However, if PARAFAC2 can be utilized it would be recommended utilizing that model over an MCR approach due to more parsimonious models as was previously mentioned.

Chapter 5

Inverse modeling

> *Essentially, all models are wrong, but some are useful.*

George E. P. Box, *Empirical Model-Building and Response Surfaces*, 1987

5.1 Purpose of modeling

There are many potential motivations for performing mathematical modeling of processes. Most applications involve modeling as a crucial step for process design, process optimization or to study possible outcomes without performing experiments in the laboratory. For example, quality by design within the pharmaceutical industry can be facilitated by modeling in order to identify critical parameters and steps in the production process [Brueggemeier et al., 2012]. In the field of SFE it is common to establish models based on experiments on a small scale in order to then scale up the process. In other cases, the researcher might just be interested in interpolation between measurement points, which could be in either space or in time, where no experimental data has been acquired. The third case, is when a deeper understanding of a process is sought. Traditional modeling, i.e., forward modeling gives the possibility of altering process parameters of a well-understood system to study the outcome.

However, it is not always the case that the process is fully understood. Therefore, inverse modeling has been one of the main tools in this thesis to study the governing phenomena of the extraction process. Inverse mod-

eling[1], also known as the inverse problem, starts with the results in order to establish the causes. Forward modeling, starts with the causes and the result is predicted [Murray-Smith, 2000]. Figure 5.1 shows conceptual difference between forward and inverse modeling.

A personal opinion is that the workflow of inverse modeling gives a systematic way of investigating and evaluating the experimentally acquired data. It can be very overwhelming to visually inspect over 30 extractograms at the same time. In the work related to microalgae over 150 extractograms were acquired and used (**Paper V**). The working process of establishing a framework for the model structure[2] provides much information. The practical work of developing models can be cumbersome. In practice many potential modeling approaches and model structures are evaluated. Thus, an understanding of the interplay between the model classes and the domain equations is formed. The journey of the model development is probably more important for the conducting researcher, rather than the knowledge that the final model actually conveys. A less scientific explanation is that an intuition is gained regarding the dataset at hand by working with it and approaching it from various angles. This intuition should not be underestimated.

5.2 Models for supercritical fluid extraction

Many mathematical models which reflect the SFE process are found in the literature. Some of them are empirical in their nature, others are based on fundamental phenomena where the parameters have a physical meaning, and other models are hybrids of the two former. There are in fact so many that no review paper has so far covered all of them. This thesis is no exception, and efforts are instead made to identify characteristics among groups of models. The works of Del Valle and De La Fuente [2006] and Huang et al. [2012] are recommended starting points for anyone who wishes to get an overview of applicable models.

Empirical models are excellent choices if a simple expression is desired, which can fit experimental data. These do, however, not reflect for example

[1]Inverse modeling is widely applied in fields such as chemical engineering, geophysics, astronomy and systems biology to mentioned a few.

[2]The same definitions are used as by Hangos and Cameron [2001]. A model structure contains the set of equations and correlations which defines the system. The set of equations may be categorized into model classes which describe e.g. solubility or partitioning. In inverse modeling these equations may contain unknown parameters which are estimated using the experimental data, this is known as model calibration. The calibrated model structure gives the well-defined process model.

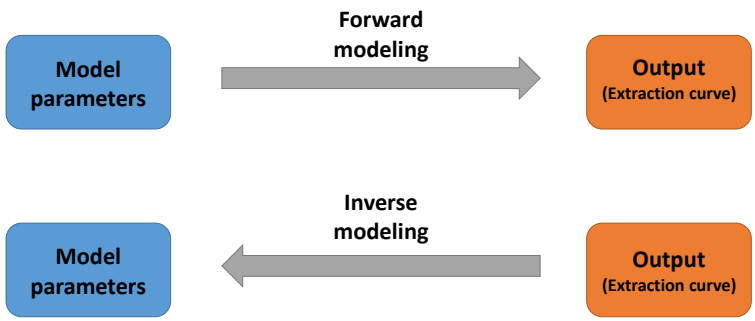

Figure 5.1: In forward modeling the governing phenomena and their parameters are known and the outcome is predicted. In inverse modeling the outcome is known and a model is derived which describe the outcome.

mass transfer kinetics or solubility. In **Paper III**, a single [Andrich et al., 2001] and a two parameter [Andrich et al., 2006] single term expression based on first-order kinetics were compared with semi-empirical and mechanistic models. These outperformed all other evaluated models by having the best fit to the experimental data.

Semi-empirical models are another class of models, which might be the most employed in the literature. These are relatively simple and are usually based on one or two fundamental concepts of limiting factors such as diffusion. The hot ball model (HBM) [Bartle et al., 1990] and the diffusion layer theory (DLT) [Veress, 1994] are examples of popular models derived with diffusion exclusively in mind. It is important to highlight that these do not consider neither solubility nor the partitioning of analytes between the extraction phase and the solid phase. If the parameters of such a model is fitted towards acquired data where the extraction is limited by solubility, this will cause a bias of the parameter. In other words, the parameter which should describe diffusivity will in fact rather describe solubility to its best capabilities. The HBM and the DLT were used in **Paper III**. For example, based on the experimental data of extracted lipids from crushed linseed, the calibrated HBMs resulted in diffusivity coefficients which spanned 16 orders of magnitude. Considering that the pressure ranged from 15 MPa to

30 MPa and the temperature ranged from 40 °C to 80 °C, these differences of diffusivities are not physically reasonable.

Other semi-empirical models are semi-continuous which divide the extraction curve into three parts [Oliveira et al., 2011]. In theory these correspond to time intervals which are limited by solubility, diffusion in the solvent within the pores and diffusion within the solid matrix [Sovová, 1994]. A few of these, for example, the broken and intact cells (BIC) model has been derived on the assumption that e.g. cells are either broken and thus the analytes are easily extractable or intact and thus the release of analyte is slower [Sovová, 2005]. Additional developments of the BIC have recently been proposed where it has been combined with the shrinking core model [Fiori et al., 2009].

Up until this point, empirical and semi-empirical models have been explicitly discussed. These are easy to set up, solve and they in general fit experimental data very well. However, they seldom give any additional information regarding the fundamental phenomena governing the SFE process. Therefore, the outcomes of new experiments cannot be predicted and the data is not useful for scaling up the process [Del Valle and De La Fuente, 2006]. A brief summary has been given, because these are widely applied in the literature but they do not resolve any of the research questions of this thesis. A more thorough discussion is given in **Paper III**.

In this work, efforts have been directed towards mechanistic models[3] with parameters of physical meaning. The channeling effects have a substantial impact on the actual starting point of developing a suitable model. Chapter 3 dealt to a great extent with the experimental implications of these. In **Papers II-III**, where lipids from crushed linseed were extracted, it was observed that the channeling effects were constant and repeatable. The extraction cell was kept packed and and the sample was stationary, thus rendering it possible to view the process as a fixed packed bed extraction (Figure 2.6). This is described by an advection term and a source term:

$$\frac{\partial c_b}{\partial t} = -u_{lin}\frac{\partial c_b}{\partial z} - \frac{3k_f(1-\epsilon_c)}{R_p\epsilon_c}(c_b - c_s) \tag{5.1}$$

where c_b is the analyte concentration in the bulk fluid, t is the time, u_{lin} is the linear velocity of the flow, z is the distance, k_f is the mass transfer resistance through the stagnant film around the particle which is also known as the

[3]In this work mechanistic models refers to models based on mathematical descriptions of mechanical or chemical phenomena or processes. The antagonistic term is empirical models based on for example linear combinations between predictors and response.

the Nerst diffusion layer, ϵ_c is the packing fraction of the column, R_p is the particle radius and c_s is the analyte concentration at the particle surface.

The particle domain is described by Fick's law of diffusion and a source term:

$$\frac{\partial c_p}{\partial t} = D_e \left(\frac{\partial^2 c_p}{\partial r_p^2} + \frac{2}{r_p} \frac{\partial c_p}{\partial r_p} \right) + \frac{1 - \epsilon_p}{\epsilon_p} \frac{\partial q}{\partial t} \tag{5.2}$$

where c_p is the analyte concentration in the particle pores, D_e is the effective diffusion, r_p is the distance in the particle, ϵ_p is the void fraction of the particle, and q is the analyte concentration in the solid phase. The partitioning between the solid phase and the extraction phase in pores were described by a partition isotherm. The many candidate partition isotherms are listed in **Paper III**. Most of them consider the solubility of the analyte $c_{solubility}$.

This modeling approach represents a packed column filled with spherical particles which are porous and the analyte is initially located in the solid. The mass transfer is driven by diffusion in the particle and by advection in the extraction column. This is known as the general rate model and is commonly used in modeling of LC and has been applied in modeling of SFE [Del Valle and De La Fuente, 2006]. It requires that the particle porosity is known or can be estimated.

In **Paper III**, an alternative model was proposed where it was assumed that the particle was one homogeneous phase through which the analytes diffused. The analogy was very much inspired by the simplicity of the HBM, and was therefore named the extended hot ball model, given by Eqs. (5.3)-(5.4).

$$\frac{\partial c_b}{\partial t} = -u_{lin} \frac{\partial c_b}{\partial z} - \frac{3k_f(1 - \epsilon_c)}{R_p \epsilon_c} \left(1 - \left(\frac{c_b}{c_{solubility}} \right)^2 \right) (c_b - c_s) \tag{5.3}$$

$$\frac{\partial c_p}{\partial t} = D_e \left(\frac{\partial^2 c_p}{\partial r_p^2} + \frac{2}{r_p} \frac{\partial c_p}{\partial r_p} \right) \tag{5.4}$$

Furthermore, the model does not have a term for the partition isotherm, leaving fewer potentially unknown parameters.

As described in Chapter 3, repeatable experiments were only achieved when accomplishing a stirring inside the extraction vessel (**Paper IV**). This had some severe implications on how to model the process, and instead a continuous stirred-tank reactor (CSTR) model was used to describe the extraction vessel. This is given by Eq. (5.5):

$$\frac{dc_b}{dt} = -\frac{Q}{V_c \epsilon_c} c_b - \frac{(1 - \epsilon_c)}{\epsilon_c} k_L a \frac{dc_p}{dt} \tag{5.5}$$

where Q is the volumetric flow, V_c is the volume of the column. The source term here corresponds to the partitioning of the analyte between the extraction phase and the solid phase and the volumetric mass transfer coefficient $k_L a$.

The drawback of a CSTR model compared to a packed bed model is related to the term describing the mass transfer between the particle and the bulk fluid. A fundamental framework exist which correlates the external mass transfer coefficient with the binary diffusivity, solvent density and viscosity, and the particle size. These correlations are given by the Reynolds number, the Schmidt number and the Sherwood number, which are provided in the supporting information of **Paper III**. For the CSTR, there are no such correlations for the volumetric mass transfer coefficient. This implies that for each system the coefficient will need to be estimated.

Much effort in this work has been directed towards incorporating the solubility of the analytes in the SCF and the partitioning between the solid phase and the extraction phase into the models . The combination of partitioning and solubility is until this day rarely considered.

In this work, the partitioning is described by a partition isotherm. These are expressed by using equations of adsorption isotherms. There are several equations which are often utilized in various applications. Many of these are presented in **Paper III** and were evaluated during the model structure estimation. The evaluated partition isotherms include a linear isotherm, Freudlich, Langmuir, Redlich-Peterson, Toth and Sips isotherms. As an example of a partition isotherm, the Langmuir partition isotherm is given in Eq. (5.6).

$$c_{solvent} = \frac{c_{solubility} K_L c_{solid}}{1 + K_L c_{solid}} \tag{5.6}$$

The analyte concentration in the solvent $c_{solvent}$ depends on the solubility $c_{solubility}$, the Langmuir equilibrium constant K_L and the concentration in the solid c_{solid}.

The recovery of analytes may not only depend on solubility and extraction time. Analytes may also be adsorbed in the solid matrix [Björklund et al., 2000, Pilorz et al., 1999]. In **Paper V** it was established that the extractability was depending on the mole fraction of co-solvent in the extraction phase. It was proposed that the extractability could be described by a sigmoidal curve as a function of mole fraction of co-solvent x, given by Eq. (5.7).

$$c_{extractable} = \frac{c_{max}}{1 + e^{\alpha(x-\beta)}} \tag{5.7}$$

Here α and β are fitting parameters and c_{max} is the concentration of the largest possible extractable amount in the solid material.

Lastly, in many equations the properties like the viscosity or the density of the SCF are needed. For pure systems these can be acquired through the NIST WebBook of thermophysical properties of fluid systems [Lemmon et al., 2016].

5.3 Inverse modeling

Inverse modeling, also known as the inverse problem, is an ill-posed problem. The lack of solution existence, solution uniqueness and instability are mentioned by Aster et al. [2013] and described as the following:

- Solution existence: No solution or model may describe the data perfectly, mainly due to crude models and measurement noise.
- Solution uniqueness: Many models may equally well describe the data. Even if there is a true model, other incorrect models may also describe the data due to rank deficiencies in the experimental data.
- Instability: In many cases small changes in the experimental data may cause large differences in the estimated model parameters.

As previously mentioned, many models and model structures are proposed in the literature. However, these are almost always calibrated using one experiment at the time. In **Paper III**, this is referred to as single calibrations. This means that each experiment will generate a set of unique estimated parameters. It has previously been argued that a single extraction curve holds too little information and that e.g. the internal mass transfer cannot be discriminated from a solubility limited extraction rate [Del Valle et al., 2012]. In **Paper III**, we confirmed that a single calibration approach was not a viable option in order to accurately estimate underlying phenomena governing the extraction process. Therefore, it is heavily argued in this thesis that a complete calibration approach should be preferred whenever possible. Published papers which primarily deals with inverse modeling of SFE very rarely describe the numerical methods and potential pitfalls during inverse modeling. These are therefore described and discussed below.

5.4 Numerical methods

Depending on the problem at hand, different numerical methods can be applied. A few categories of problems can be identified. Partial differen-

tial equations (PDEs) can rarely be solved analytically and do often need to be solved through numerical methods. These are usually restructured into a system of ordinary differential equations (ODEs) through so called discretization. Solving ODEs also requires a specific set of numerical solvers. Finally, non-linear optimization problems, e.g., parameter optimization is another set of problems encountered in this work. **Paper III** visualizes the various levels of problems which need to be solved through numerical methods.

It would be possible writing an entire thesis regarding only numerical methods suitable for simulating a packed bed. Much inspiration has been acquired from previously published works regarding numerical simulation of LC.[4] From a mathematical point of view, the problems are very similar. The exceptions are that there is no impulse, i.e., an injection, rendering simulations much easier and that the initial concentrations inside the particles are not zero. A few examples of LC modeling and inverse modeling are given in the following references [Borg et al., 2013, 2014, Karlsson et al., 2004, Nilsson et al., 1999].

5.4.1 Simulation of models

Starting with the domain equations, solving PDEs is an art of its own. It is important to realize that many methods exist, e.g., finite elements, boundary elements, spectral methods to mention a few [Farrashkhalvat and Miles, 2003]. In this work the finite volume method (FVM) was utilized to solve the PDEs describing the extraction process in a packed bed. The FVM is beneficial because it is relatively easy to implement and it is a conservative method, i.e., all mass is maintained, which is not nessecarily the case with for example finite differences or the finite element method [Farrashkhalvat and Miles, 2003].

FVM is principally based on calculating values of discrete positions within the domain, i.e. the extraction column or the particle. The discrete positions form a mesh which represents the geometry, where each node point represents the volume around it (Figure 5.2). This results in that the PDEs are converted into a system of ODEs. Various discretization schemes exist which differ in numerical stability, accuracy and calculation speed. The schemes differ in which and how many neighboring cells are taken into account in the approximation. Higher order schemes increases

[4]Many of the practical modeling and numerical issues are discussed in the Ph.D. thesis of N. Borg, *Modeling and Calibration of Preparative Chromatography*, Lund University (Media-Tryck), Lund, 2013.

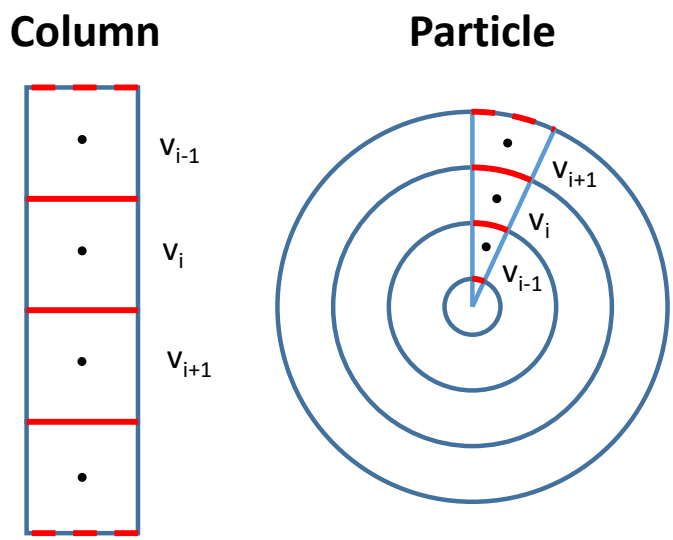

Figure 5.2: The principles of one dimensional discretization using the FVM. The dots represent the nodes that corresponds to the mean analyte concentration of each volume. The red lines show the interfacing area between the cells through which there is a flux of analyte. The dashed lines at the end of each domain represents the boundaries. Note that the area in the column discretization is constant in the column mesh, while it depends on the coordinates in the particle.

accuracy but might also induce instability resulting in oscillating solutions [Losasso et al., 2006]. The suitable approximation scheme is very problem-dependent. In this work a two-pointbackward difference scheme was used for the first-order PDEs and a three-point central difference scheme was used for the second order PDEs. The discretization scheme assumed that the column domain was completely homogenous in the radial dimension and that the particle was homogenous over its spherical coordinates. By doing so a one dimensional method of lines is acquired for both domains, reducing the number of nodes and rendering simulations reasonably fast.

Discretization of the column was performed in one spatial dimension with equal meshing distance, thus equal volumes and interfacing areas. The advection term was discretized according to Eq. (5.8):

$$\frac{\partial c_v}{\partial t} = \frac{c_v - c_{v-1}}{h} \tag{5.8}$$

where c_v denotes the analyte concentration in the cell volume, the cell height h corresponds to the mesh size of the column.

The discretization of the particle is not as straight-forward as for the column domain as seen in Eq. (5.9). This is due to the spherical symmetry of the particle.

$$\frac{\partial c_v}{\partial t} = \frac{\left(A_i \frac{c_{v+1} - c_v}{\bar{r}_{v+1} - \bar{r}_v} - A_{i-1} \frac{c_v - c_{v-1}}{\bar{r}_v - \bar{r}_{v-1}} \right)}{V_v} \tag{5.9}$$

In Eq. (5.9) A corresponds to the interfacing area through which the analyte is diffusing (Figure 5.2). The simplest ways to generate an even distribution of the mesh is to utilize equal distance between nodes or to use equal volumes of each node. Equally sized meshing volumes resulted in much faster calculations due to better numerical stability and enabled a more coarse mesh which further speeded up the calculations (Figure 5.3).

From a numerical point of view, and out of personal experience, the approximations of the boundaries are the most difficult in order to acquire an accurate numerical approximation. From Eq. (5.10), the analytical expression for the boundary conditions at the particle surface can be derived and is given by Eq. (5.12).

$$k_f A \left(c_b - c_s \right) = D_e A \left. \frac{\partial c_p}{\partial r} \right|_{r=R} \tag{5.10}$$

$$k_f A \left(c_b - c_s \right) = D_e A \frac{c_s - c_{p,r=max(r)}}{R_p - r_{r=max(r)}} \tag{5.11}$$

$$c_s = \frac{k_f c_b \left(R_p - r_{r=max(r)} \right) + D_e c_p}{D_e + k_f \left(R_p - r_{r=max(r)} \right)} \tag{5.12}$$

In **Paper III**, a distribution term K_d was evaluated in some single calibration models. The boundary condition at the particle surface was given by the following expressions:

$$k_f A \left(c_b - \frac{c_s}{K_d} \right) = D_e A \left. \frac{\partial c_p}{\partial r} \right|_{r=R} \tag{5.13}$$

$$c_s = \frac{k_f c_b \left(R_p - r_{r=max(r)} \right) + D_e K_d c_p}{D_e K_d + k_f \left(R_p - r_{r=max(r)} \right)} \tag{5.14}$$

In the case where the extraction is described by a CSTR model, no discretization is needed, as this is already an ODE (Eq. (5.5)). Discretized

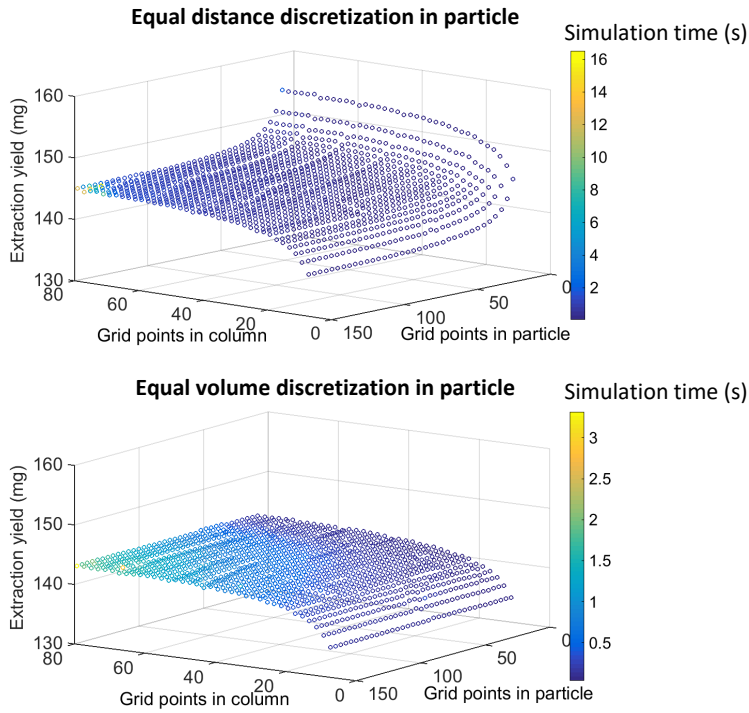

Figure 5.3: Two different discretization methods of the particle were evaluated. Either using equal distance between the grid points (top) or equal volumes of each grid point (bottom). The effect of the mesh coarseness of both the column and the particle was then evaluated by running simulations of a SFE process. Finer meshes generally generate a more accurate numerical solution, however, at the cost of longer simulation times. The accuracy of the solution was evaluated by calculating the extraction yield at 60 minutes. Ideally, this value should be the same, independent of the numerical solution method. It can be observed that the numerical simulations break down by using fewer grid points than 20 in the column. Equal volume discretization in the particle improved both the simulation time and reduced the number of grid points needed.

systems were solved using a numerical solver for stiff ODEs problems using a variable order method, namely the *ODE15s* in MATLAB. The CSTR problems were solved using the *ODE45* function in MATLAB. Numerical solvers are readily available in many commercial and open-source softwares and are therefore not thoroughly discussed here. The interested reader is referred to [Beers, 2006].

5.4.2 Calibration of model structures

For a given model structure the unknown parameters need to be estimated. This is usually performed by minimizing an objective function. The objective function is usually based on least-squares, or equivalent measurements, between the simulated extraction and the experimentally acquired extraction curves. In this work the root mean square error (RMSE) has been utilized, because the least-squares can result in huge numbers and the RMSE is of the same size as the original data rendering the results more intuitive.

The numerical approach to optimization problems is rather complicated and depends on the problem. It is therefore difficult to give any general recommendations on which method to use. Factors to consider are the dimensionality of the problem, the computational cost of performing simulations, multiple minima of the objective function and how the simulated data is acquired.

For example, gradient-based search methods like quasi-newton or the interior point algorithms are extremely efficient at finding an optimum solution if there is only one minimum and if the objective function is smooth [Fu, 1994]. If the objective function is based on simulated data which is noisy due to poor numerical accuracy, gradient-based solvers may fail [Fu, 1994]. If there are several minima, these solvers will likely get caught in local minima rather than the global minimum. In the literature, the gradient-based algorithms are still used as the main numerical optimization solvers for inverse modeling of SFE.

Many alternatives exist, e.g., using a gradient-based solver with multiple starting points, non-gradient-based solvers using mesh adaptive direct search (MADS) algorithms [Audet and Dennis Jr., 2007] or metaheuristic search methods like the genetic algorithm (GA) and the particle swarming optimization (PSO) algorithm [Blum and Roli, 2003]. These can also be combined by initializing with one numerical solver and performing the final fine tuning using another.

In **Paper III**, metaheuristic methods were evaluated and employed because of various reasons. First one being that MADS did not converge

towards a reasonable solution using any tried settings. The objective function was computationally expensive, i.e., between a few seconds up to one minute for simulating 29 SFE experiments depending on which parameters were evaluated. By performing evaluations in parallel, the total calibration time can be substantially reduced. Metaheuristic solvers are usually based on populations which are guided by candidate solutions based on the evaluations with the best fit from a previous iteration [Blum and Roli, 2003]. These calculations were performed on a cluster of computers with approximately 60 CPU cores. Thus, each member of the population can be put in a queue and evaluated in parallel by one of the cores.

Both GA and PSO are relatively efficient at avoiding getting caught near local minima. Both are based on populations which are initially scattered over the parameter space. Candidate solutions, i.e., members which resulted in the best objective function evaluation, influence the population in which parameter values should be evaluated in the next iteration. This is beneficial when large parts of the parameter space result in very similar objective function outputs. This is difficult to visualize if more than two or three parameters are estimated, as was the case in **Paper III**. An example is presented in Figure 5.4, where only two parameters were estimated using the single calibration method. It bears resemblance with the Rosenbrock function which is a typical benchmarking function for numerical optimization solvers, as it is difficult to numerically find the global minimum [Shang and Qiu, 2006]. This gives some indication of the problem difficulty.

In **Paper III**, it was found by trial and error using various settings that the PSO algorithm was overall performing better by reaching the same solution at a fewer objective function evaluations.

The calibrations of the model structures in **Paper V**, were on one hand much simpler because the model consisted of only one ODE per analyte evaluated. This meant that all experiments together could be simulated in less than half of a second. Also all simulations were performed with higher numerical accuracy due to the simple nature of the problem, thereby enabling the use of gradient-based solvers for calibrations. On the other hand, solubility and partition related parameters were determined using complete calibration while the volumetric mass transfer coefficient was determined by single calibrations. A schematic of the methodology is presented in the supporting information of **Paper V**.

For the analyte-specific model structure calibration an extensive search using Latin hypercube sampling (LHS) was used prior to initializing the MADS algorithm using the best candidate as a starting point. At each iteration, a parameter estimation of k_La was performed for each experiment.

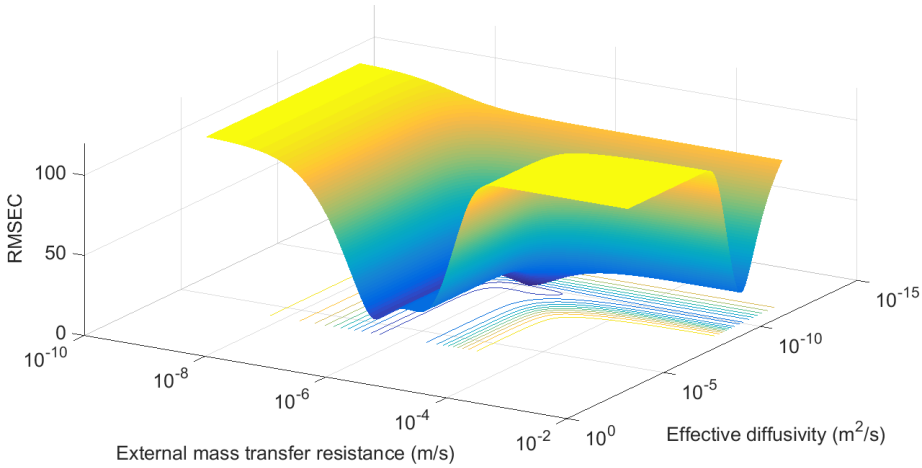

Figure 5.4: Example of an objective function when performing a single calibration of a mechanistic model. The external mass transfer resistance, k_f and the effective diffusivity D_e are estimated. The objective function has a valley which is very flat in nature, rendering the global minimum difficult to find.

This was performed using an interior point algorithm [Byrd et al., 1999], due to the smooth objective function with only one minimum (Figure 5.5).

During model calibration some combination of parameter values may give rise to unrealistic phenomena. The result could be a simulation where the solubility is extremely high, e.g., several kg/L. These simulations are usually time demanding or may not even converge. Most of these issues can be avoided by simply not simulating models with an unrealistic solubility estimation and instead returning a relatively high objective function value. This will resemble a soft constraint.

5.4.3 Estimation of model structures

Considering the many and the diverse models which are potentially useful within each model class, it is a challenge finding the correct model structure. As is visualized in **Paper III**, there are many expressions which are able to describe e.g., solubility or diffusivity over the evaluated extraction conditions. In the available literature the model structure is arbitrarily chosen.

The selection of equations within each model class which results in the model structure is a very difficult combinatorial problem. Unfortunately,

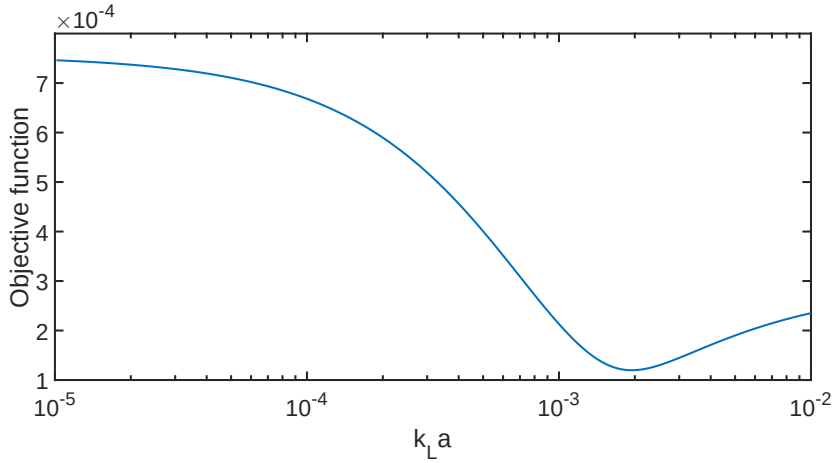

Figure 5.5: The characteristic objective function when estimating the volumetric mass transfer coefficient (**Paper V**).

a true minimum can only be assured by an exhaustive search among all possible combinations [Nievergelt, 2000]. This is unfortunately not feasible as the number of possible combinations can very well exceed thousands or millions of model structure candidates.

In **Paper III**, two systematic approaches were evaluated. One based on GA and another by performing an initial LHS search before evaluating one model class at the time. Both approaches managed to find suitable model structures, however, the GA required more function evaluations. Interestingly, by inspecting all the top candidate model structures evaluated, certain trends could be observed within the different model classes. For example, it was noted that the top model structure candidates consistently contained the Sovova solubility model [Sovová et al., 2001] and the Toth partition isotherm. No such trend was observed regarding the model classes relating to mass transfer. This suggests that the solubility and the partitioning might be the most important starting point when performing inverse modeling.

5.5 Forward modeling

A lot can be learned about SFE through inverse modeling. When a suitable model structure is found and has been calibrated to obtain the final model, additional research questions may be answered. For example, based on the obtained model of lipid extraction from linseed, how small does a particle

need to be before the intra-particle mass transfer can be disregarded? This particular question was answered by running several simulations of SFE with various particle sizes (Figure 5.6). Through the simulations it was concluded that reducing the particle size below 0.11 mm had virtually no effect on the extraction rate.

This knowledge became essential in performing inverse modeling of analytes from microalgae (**Paper V**). The size of the microalgae was not measured, but most microalgae range from a 5 to 50 μm in size [Uduman et al., 2010]. It was therefore deemed that the intra-particle mass transfer could be disregarded, and thus only two phases were considered.

Other tests are possible to assess the model structure. For example, the significance of the partitioning during the extraction can be evaluated. This is shown in the supporting information of **Paper V**, which indeed suggests that partitioning is an important phenomena.

An SFE application could be optimized if a model is acquired. This was, however, not evaluated in this work. This requires that the criteria of which makes an extraction desirable are known. For example, if intra-particle mass transfer phenomena are substantial, it might be beneficial starting out with a high flow rate which decreases over time in order to make an extraction time efficient but yet lowering the CO_2 consumption. What makes an extraction method desirable will always be a trade-off of many factors and very dependent on the application and preferences of the user.

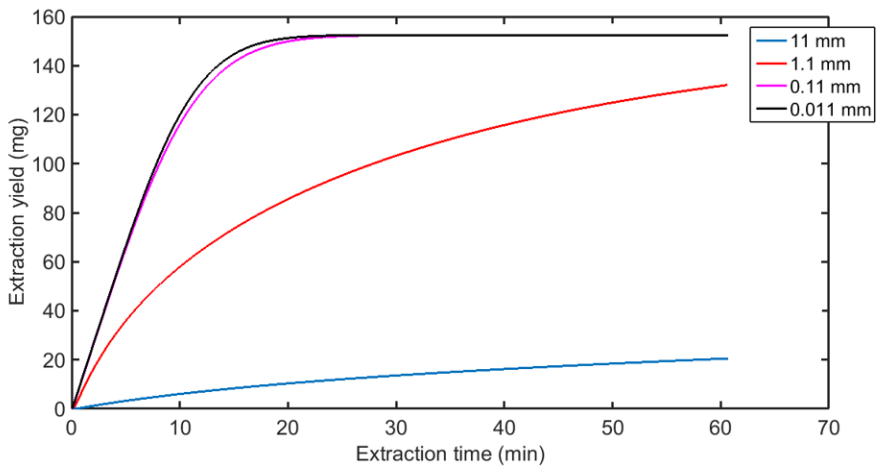

Figure 5.6: Simulated extraction curves using various particle sizes of crushed linseed. The simulation was performed using the top model candidate acquired in **Paper III**. Extraction conditions were 30 MPa, 353 K, 2 mL/min flow rate of neat CO_2. The sample amount was 1.5 g of crushed linseed. The simulations suggest that reducing the particle size to smaller than 0.11 mm in diameter has a very limited effect on the extraction rate.

Chapter 6

Design of experiments

> *It is the weight, not numbers of experiments that is to be regarded.*

Isaac Newton

Up until this part of the thesis it has been discussed how to perform SFE, measurements, data treatment and inverse modeling. However, which experiments to perform has not been covered so far. It was mentioned already in Chapter 1 that it is counter-intuitive describing the design of experiments (DOE) last. For most analytical chemists probably the most familiar work flow starts with the DOE, continuous with the chemical analysis and proceeds with the data analysis.

However, if the overall methodology involves inverse modeling, then it is critical to understand the model structure and thereby the important underlying phenomena governing the extraction process. This is further complicated by the fact that the model structure might not initially be known, and is therefore estimated during the inverse modeling. Furthermore, no investigation or development has been done in this work regarding what an optimal DOE looks like. This is the reason why this topic is discussed last and will mainly include general practical considerations, limitations and possible future work.

It should also be noted that this topic has not been discussed in the context of SFE before. Although no solution to the issue is presented below, perhaps a more pragmatic approach can still be utilized. In Chapter 4 and in Chapter 5 the concept of rank is discussed. This very much relates to the DOE. Ideally, there should be enough systematic variation in the data to be able to distinguish the phenomena of interest. For example, if the solubility is the main phenomenon of interest, ensuring a good coverage of various

densities may be a good starting point. Furthermore, it was important to include several experiments using varying mole fractions of co-solvent in order to derive a usable MCR model for establishing individual extraction curves (**Paper IV**). Otherwise the extraction profiles of the analytes covaried too much to be successfully resolved.

Randomized run order of the SFE experiments is essential. For example, if a series of experiments are conducted in an order of increasing pressure and density, the parameters may be affected by a systematic error. Recall the issue of analyte degradation which was discussed in Chapter 3. If the analytes in the stock solution degrade over time, this will affect the estimated extracted amount. Thus, the response for each injection will decrease for each experiment and the estimated yield will incorrectly increase. By using a randomized run order of the experiments, this error will present itself as a random error rather than a systematic [Montgomery, 2009].

6.1 Parameters

An abundance of literature is available where extraction conditions have been optimized for a particular application, here followed by a few examples of available published works [Chi et al., 2016, Mackela et al., 2015, Martins et al., 2016, Pang et al., 2015, Przygoda and Wejnerowska, 2015, Sodeifian et al., 2016]. These works generally use response surface methodology (RSM) with the process parameters as decision variables, which are correlated with the extraction yield using linear or quadratic relationships [Sharif et al., 2014]. The process parameters here refer to pressure, temperature, CO_2 and co-solvent flow rate. This approach offers an easy and intuitive way of optimizing an extraction method.

The designs used in RSM focus on minimizing the parameter estimation error when fitting the model to the experimental data. It should here be remembered that the DOE assumes that the correlation between the process parameter and the extraction yield is either linear or quadratic. This is usually true for most systems and problems, when only investigating a very limited range of parameters [Montgomery, 2009]. However, based on the non-linear nature of models and the extraction curves, the characteristics of extraction curves can most likely not be reduced to linear expressions if a larger design space is evaluated.

Perhaps it is more correct to design an experimental plan based on the parameters which directly relate to phenomena which are the scope of the study. For example, pressure is not directly related to solubility, however, rather indirectly related through the density of the SCF. Since it is in fact the

density which is of interest, perhaps it is wise to also design experiments accordingly. In **Paper III** the 3-level full factorial DOE was based on the process parameters, i.e. pressure and not density, resulting in a $scCO_2$ density of less than 800 g/L in a majority of the experiments. According to e.g. the Chrastil solubility correlation [Chrastil, 1982], the solubility increases exponentially along with density. In **Paper III** this meant that the extraction rate was rather low due to poor solubility when the density was comparably lower. It was also observed that the model fit was worse at higher densities. It is possible that performing more experiments at a higher density would have been beneficial.

In **Paper V** the DOE was based on the density rather than the pressure. Of course, it means an additional step of estimating or retrieving density data in order to construct such a design. A visualization is given in Figure 6.1, how the two different approaches to the DOE affects the design space of density and temperature.

6.2 Experimental constraints

Unfortunately, not all parameters can be evaluated because of mainly experimental limitations. The maximum pressure and temperature will depend on the least resistant component of the SFE system. Naturally, the model validity can only be assured when kept within the parameter design space. Some SFE processes are operated above 40 MPa [Clifford, 1999], however, because of instrumental limitations these conditions have not been studied in this work. Pressure and temperature limitations are given mainly by the optical flow cell. Pressure limitations are given mainly by the extraction vessel but also the BPR and the valves of the used system in this work.

Another slight issue is that an unsymmetrical DOE will be acquired if the density is regarded as a studied parameter rather than pressure. This is caused by the constraints in pressures which can be used by the equipment. Because as the temperature is increased, a higher pressure is needed to maintain a constant density of the SCF.

In this work, syringe pumps were exclusively used to pump the CO_2. These are filled to a fixed volume and the experiments need to be interrupted when a refill is needed. This introduces yet another constraint on how long the extraction times can be.

Other constraints may appear which are not easily predicted. In **Paper IV** it was noticed that flow rates below 1.0 mL/min did not generate enough stirring inside of the extraction vessel to ensure that channeling effects were minimized.

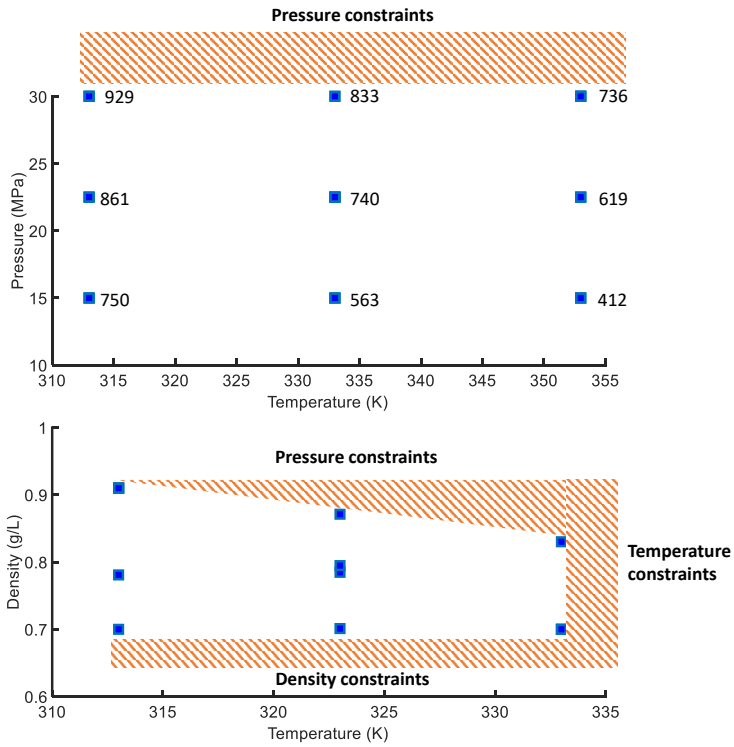

Figure 6.1: The DOEs used in **Paper III** (top) and **Paper V** (bottom). The densities of neat $scCO_2$ (g/L) are given as values next to each point in the top figure. In this case, most experiments were performed at densities around 750 g/L and experiments with higher densities than 800 g/L were few. The pressure constraints due to equipment limitations are easily met by the design. The DOE for the microalgae was instead based on density rather than pressure. An additional temperature constraint appeared due to degradation of analytes at higher temperatures.

6.3 Optimal experimental design

Ideally, the model building should be based on an iterative cycle [van Riel, 2006]. In the works of this thesis only one cycle of measurements, data processing and inverse modeling was performed as visualized in Figure 1.1. This enables a revision of previously defined hypotheses and introduces the opportunity to perturb the extraction system in ways which may facilitate a more critical approach to the derived models. Optimal designed experiments is the methodology that decides which experiments should be performed at each subsequent cycle in order to address uncertainties in the model [van Riel, 2006]. Without going into greater depth, methods based on the so called Fischer information matrix (FIM) have been proposed within systems biology to iteratively determine which time points during a process should be measured in order to most accurately determine unknown parameters [Baltes et al., 1994, Kutalik et al., 2004]. This might be useful if for example on-line measurements do not offer enough selectivity and off-line measurements using chromatographic techniques become necessary. The FIM is a measure of information content in the data and can therefore be used to estimate the accuracy of the estimated parameters [van Riel, 2006]. It has been shown in several applications that this approach has generated models with better parameter estimates [Faller et al., 2003, Gadkar et al., 2005, Kreutz and Timmer, 2009, Rodriguez-Fernandez et al., 2006].

None of these concepts have been employed in DOE for SFE. Until then, the pragmatic approach of deciding which conditions the extractions should be performed may be made based on experience. However, it may be a way forward in determining which experiments to perform in the laboratory. This would certainly help to gain as much information as possible from each conducted SFE experiment.

Chapter 7

Conclusions

> *I have always seen modeling as a stepping stone.*

Tyra Banks

7.1 Concluding remarks

In this thesis two sample matrices have been the main subject of study, namely crushed linseed and microalgae. However, the methodology developed in the framework of this thesis is more universal. It is shown that a single extraction experiment does not contain enough information to reliably determine underlying phenomena which affect the SFE process. A complete calibration methodology is needed to properly study extraction kinetics. In this work such a methodology is presented for SFE.

A complete calibration methodology is more demanding in terms of data acquisition because more experiments are needed. Here in-line and on-line measurements substantially reduce the manual labor in the laboratory. A temporal resolution is achieved which would not be possible by collecting fractions and performing off-line measurements. It is also shown that the precision is improved compared to doing off-line analysis.

Off-line measurements still provide incomparable selectivity compared to in-line measurements. A rapid SFC method was developed for separation of carotenoids from microalgae.

It is also shown that the traditional interpretation of extraction curves, where a constant extraction rate corresponds to solubility limitations and a diminishing extraction rate corresponds to intra-particle diffusion limita-

tion, might not always be correct. Partitioning of the analyte between the solid matrix and the extraction phase may also cause these characteristics.

Channeling effects were a reoccurring issue throughout all studies in this thesis. This is particularly interesting because this is rarely mentioned the works of others. This is crucial to consider as it will both introduce a systematic error to chemical analysis and cause a bias of estimated parameters during inverse modeling if left unnoticed.

7.2 Future work

The presented work at hand highlights many potential issues and limitations. The channeling effects still remain a challenge, and alternative ways to avoid these should be investigated. The experimental conditions have also been limited by the lack of commercially available equipment which can withstand much higher pressures and temperatures. As this development will progress, it will enable inverse modeling of larger design spaces. Computational power and computationally more efficient numerical methods would certainly facilitate inverse modeling. Finally, much work remains in determining which SFE experiments should actually be performed in the laboratory in order to study extraction kinetics. Some inspiration, potential ideas and reflections are, however, presented in Chapter 6.

In this work a top-down methodology has exclusively been used. Questions might arise if this approach is superior to a bottom-up approach, where separate experiments are performed to study solubility, diffusion and so on. I believe that the way forward for the research field is to perform research using both approaches. Parameters can then be verified by comparison and established parameters may lower the number of parameters for calibration during inverse modeling. Nonetheless, it remains clear that modeling offers a versatile stepping stone for fundamental research.

This thesis has for sure left some loose ends as well. In **Paper II**, a substantially higher yield of lipids was acquired compared to the Soxhlet method. The reason and whether there is really a difference in composition remains unknown. In Paper V, it was noticed the extractability depended on the co-solvent fraction in the extraction phase. However, what actually causes the inhibition of these fractions of analytes is also left for future research.

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[1]Inga nämnda, inga glömda.

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