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Extraction and Chromatography of Bioactive Compounds in Complex Samples using Supercritical CO_2 Technology

SAID AL-HAMIMI | CENTRE FOR ANALYSIS AND SYNTHESIS | LUND UNIVERSITY



Extraction and chromatography of bioactive compounds in complex samples using supercritical CO₂ technology

Said Al-Hamimi



DOCTORAL THESIS which, by due permission of the Faculty of Science, Lund University, Sweden, will be defended at lecture hall B, Kemicentrum, Sölvegatan 39 A, Lund, Sweden on Friday, October 12, 2018, at 9:15 AM

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Extraction and chromatography of bioactive compounds in complex samples using supercritical CO₂ technology

Said Al-Hamimi



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"Desire has no history..." Susan Sontag

To my parents, brothers and sisters, for their support and encouragement.

To my wife, my son and my daughter for their endless love and inspiration.

Abstract

Bioactive compounds found in plants have been of interest to man since ancient times. These compounds have the ability to modulate the metabolic processes in our bodies, which suggests that they may promote better health. Bioactive compounds vary in their chemical structure, polarity, stability and biological activity. This diversity makes the study of bioactive compounds challenging from the perspective of analytical chemistry. The extraction of bioactive compounds using conventional solid–liquid extraction (SLE) is slow due to mass transfer limitations. While increasing the temperature speeds up the mass transfer, it also leads to degradation and oxidation. Supercritical CO_2 (ScCO₂) extraction offers high mass transfer at low temperature, but it has selective solubility towards nonpolar compounds.

This thesis describes the development of techniques and methods for the extraction and chromatographic analysis of bioactive compounds leading to improvements in mass transfer, solubility and resolution, using ScCO₂ technology. Ultrahighpressure supercritical fluid extraction improved the solubility and extractability of oil from moringa seeds due to an increase in the density of the solvent. Extraction at 80 MPa increased the amount of oil extracted by about 30% in a short time, compared to extraction at 40 MPa. The selectivity was also affected, as higher content of polyunsaturated fatty acids and some phospholipid species were detected in the oil extracted at 80 MPa. CO₂ expanded liquid extraction (CXLE) combined enhanced mass transfer and high solubility, which resulted in a high extraction rate. The addition of CO₂ to a liquid organic solvent decreased the viscosity and changed the solubility parameters. CXLE showed a 10 times faster extraction rate of cisverbenol from Boswellia sacra resin compared to supercritical fluid extraction (SFE) and SLE. A combination of sonication and CXLE improved the solubility and extractability of the oil from different berry seeds. Sonication increased the amount of oil extracted using CXLE 3-fold. The composition of the oil obtained using CXLE showed significant increases in the levels of phospholipids and glycolipids compared to the oil obtained by SLE.

A method of supercritical fluid chromatography (SFC) was developed based on a Diol column, which showed the highest peak height, a small peak width and high resolution between and within lipid classes. Stationary phases with a β -amino alcohol ligand showed a very strong retention of the zwitterionic lipids with terminal primary amines such as phosphatidylethanolamines. The sensitivity of mass

spectrometry (MS) was found to be dependent on the composition of the SFC mobile phase. Optimization of the ion source settings in MS is important to achieve a compromise between the detection sensitivity of early and late eluting peaks.

The impact of bioactive compounds in lingonberries on metabolites in plasma was also investigated. The results showed that the intake of lingonberries could improve the liver function and decrease the effects of high-fat diet. The intake of lingonberries could also prevent the formation of metabolites associated with an unhealthy phenotype such as sphingomyelins by decreasing the level of serine.

Popular scientific summary

Medical doctors and nutritional specialists recommend eating healthy foods including fruits and vegetables. Cosmetic experts also recommend the use of cosmetic products obtained from natural products. The reason for this is that natural products from plants contain chemical compounds that can promote health and protect our bodies from several diseases. These chemical compounds are called bioactive compounds. They act as antioxidant, antimicrobial and anti-inflammatory agents in our bodies. These compounds have diverse structures and are found among thousands of other compounds in plants. Their properties also differ: some are fat-like while others are water-like. Some of these compounds are unstable, and are sensitive to heat and light, making their extraction and analysis difficult.

In the research described in this thesis, extraction methods were developed in an attempt to obtain high solubility, high recovery in a short time, and selectivity to the desired compounds, while being sustainable and unharmful to the environment. Ultrahigh-pressure supercritical fluid extraction was used to extract oil from moringa seeds. Applying a higher pressure increases the density of the CO₂, which in turn increases the dissolution and extraction properties of the fluid, thus increasing the extraction rate. The amount of oil obtained at 80 MPa was 400 mg/g seeds, while it was only 278 mg/g seeds at 40 MPa. The oil obtained at 80 MPa also had a high content of polyunsaturated fatty acids and some polar lipids belong to phospholipid class.

CO₂ was also added to the liquid solvent in a technique called CO₂ expanded liquid extraction (CXLE) to improve the mass transfer and solubility, instead of using high temperature. This technique was used to obtain aroma compounds from plant resin (*Boswellia sacra*). CXLE was found to extract the aroma compounds 10 times faster than supercritical fluid extraction (SFE). Combining ultrasound treatment with CXLE led to high and fast recovery of oil from various berry seeds compared to CXLE alone. Ultrasound improved the mass transfer and also allowed the solvent to penetrate further into the sample, increasing the solubility and subsequently extracted amount.

The results of supercritical fluid chromatography (SFC) revealed that some lipid species can interact very strongly with the stationary phases, which causes broadening of the peaks and a reduction in the resolution and efficiency. The presence of β -amino alcohol on the stationary phases caused distortion of the peaks belonging to lipids having terminal primary amines. The Diol stationary phase

showed the highest peak height and resolution of the lipid species. The method developed can resolve more than 15 lipid classes within 11 min.

Lingonberries are rich in bioactive compounds, especially polyphenols. Intake of lingonberries can reduce the impact of a high-fat diet and improve liver function. It has been suggested that lingonberries can alter the formation of unhealthy components in the body, such as sphingolipids. High levels of sphingolipids are associated with the development of obesity and diabetes.

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

- I. Carbon dioxide expanded ethanol extraction solubility and extraction kinetics of α-pinene and cis-verbenol
 Said Al-Hamimi, Alícia Abellan Mayoral, Larissa P. Cunico, Charlotta Turner
 Anal. Chem. 2016, 88, 4336–4345
- II. Screening of stationary phase selectivities for global lipid profiling by ultrahigh performance supercritical fluid chromatography
 Said Al-Hamimi, Margareta Sandahl, Marina Armeni, Charlotta Turner, Peter Spégel
 Journal of Chromatography A, 2018, 1548, 76–82
- III. Ultra-high pressure supercritical fluid extraction and chromatography of oil from *Moringa oleifera* and *Moringa peregrina* seeds Yannick Nuapia Belo, Said Al-Hamimi, Luke Chimuka, Charlotta Turner Manuscript
- IV. A fast and green extraction method for berry seed lipid extraction using CO₂ expanded ethanol combined with sonication
 Said Al-Hamimi, Charlotta Turner Manuscript
- V. In-line UV-vis absorption spectroscopy for quantification of carotenoid and flavonoid components in berry pomace using continuous CO₂ expanded liquid extraction
 Said Al-Hamimi, Larissa P. Cunico, Victor Abrahamsson, Charlotta Turner Manuscript
- VI. Alterations in the plasma metabolite profile associated with improved hepatic function and glycaemia in mice fed lingonberry-supplemented high-fat diets
 Said Al Hamimi, Lovisa Heyman-Lindén, Merichel Plaza, Charlotta Turner, Karin Berger, Peter Spégel
 Mol. Nutr. Food Res. 61, 3, 2017, 1600442

Author's contributions

Paper I: I planned and designed the experiment together with Charlotta Turner. I performed the experiment together with Alícia Abellan Mayoral. Larissa P. Cunico performed the data modelling. I wrote the manuscript together with Larissa P. Cunico and Charlotta Turner.

Paper II: SA and PS planned and designed the experiment. SA performed the experiment and data analysis. MA, MS and PS assisted in the data analysis. PS, MS and CT revised the manuscript.

Paper III: SA, YB and CT planned and designed the experiment. SA and YB performed the experiment and wrote the first draft of the manuscript. CT and LCH assisted in the data analysis. PS and CT assisted in writing the manuscript.

Paper IV: SA and CT planned the work. SA designed the experiment, analysed the data and wrote the manuscript. CT assisted by revising the manuscript.

Paper V: SA, LC and CT planned and designed the experiment. SA performed the lab work. LC and VA carried out the data analysis and modelling. SA wrote the first draft of the manuscript. CT revised the manuscript.

Paper VI: KB and PS conceived the study. SA and PS planned and designed the experiment. SA performed the metabolite profiling and data analysis, assisted by PS. MP performed the phenolic analyses. LH performed the animal studies. SA and PS wrote the first draft of the manuscript, and CT assisted in writing the manuscript.

SA: Said Al-Hamimi, LC: Larissa P. Cunico, AM: Alícia Abellan Mayoral, MA: Marina Armeni, MP: Merichel Plaza, LH: Lovisa Heiman, YB: Yannick Nuapia Belo, VA: Victor Abrahamsson, MS: Margareta Sandahl LCH: Luke Chimuka, KB: Karin Berger, PS: Peter Spégel and CT: Charlotta Turner

Related publications not included in this thesis

Composition and physicochemical properties of dried berry pomace Anne-Marie Reißner, **Said Al-Hamimi**, Amparo Quiles, Carolin Schmidt, Susanne Struck, Isabel Hernando, Charlotta Turner, Harald Rohm Journal of The Science of Food and Agriculture (**2018**) DOI:10.1002/jsfa.9302

Stephen Kwao, **Said Al-Hamimi**, María Elena Vicente Damas, Allan G. Rasmusson, Federico Gómez Galindo; Effect of guard cell electroporation on drying kinetics and aroma compounds of Genovese basil (*Ocimum basilicum* L.) leaves; Innovative Food Science and Emerging Technologies 38 (**2016**) 15–23

Estelle Larsson, **Said Al-Hamimi**, Jan Åke Jönsson; Behaviour of nonsteroidal anti-inflammatory drugs and eight of their metabolites during wastewater treatment studied by hollow fibre liquid phase microextraction and liquid chromatography mass spectrometry; Science of the Total Environment 485–486 (**2014**) 300–308

M. S. Khan, M. K. Al-Suti, H. H. Shah, **S. Al-Hamimi**, F. R. Al-Battashi, J. K. Bjernemose, L. Male, P. R. Raithby, N. Zhang, A. Köhler and J. E. Warren; Synthesis and characterization of platinum (II) di-ynes and poly-ynes incorporating ethylenedioxythiophene (EDOT) spacers in the backbone; Dalton Trans., **2011**, **DOI:**10.1039/C1DT11010A

Márta Kubovics, **Said Al-Hamimi**, György Huszár, Kinga Komka, Charlotta Turner, Edit Székely; Preparation and analysis of polar hawthorn berry extracts, industrial application in poultry processing; Submitted to Periodica Polytechnica Chemical Engineering (under revision)

Mingzhe Sun, **Said Al-Hamimi**, Margareta Sandahl, Charlotta Turner; Coupling extraction and chromatography in an on-line comprehensive two-dimensional system for dynamic extraction kinetics studies; Manuscript

Alicia Gil-Ramirez, **Said Al-Hamimi**, Oskar Rosmark, Oskar Hallgren, Anna-Karin Larsson-Callerfelt, Irene Rodríguez-Meizoso; Efficient methodology for the analysis of lipids in porcine pulmonary artery by supercritical fluid chromatography coupled to mass spectrometry; Manuscript

List of abbreviations

BEH:	ethylene-bridged hybrid
CXE:	CO ₂ expanded ethanol
CXL:	CO ₂ expanded liquid
CXLE	CO ₂ expanded liquid extraction
DOE:	design of experiments
ESI	Electrospray ionization
FFA	Free fatty acids
GC	Gas chromatography
GXL:	gas-expanded liquid
HPLC:	high performance liquid chromatography
HSP	Hansen solubility parameter
MS:	mass spectrometry
OPLS:	orthogonal partial least squares
PCA:	principal component analysis
PHW:	pressurized hot water
PLE:	pressurized liquid extraction
PLS	Partial least squares
QTOF-MS	quadrupole time-of-flight mass spectrometer
ScCO ₂ :	supercritical carbon dioxide
SFC:	supercritical fluid chromatography
SFE:	supercritical fluid extraction
SLE:	solid–liquid extraction
UHPLC	Ultra-high performance liquid chromatography
UHPSFC:	ultrahigh performance supercritical fluid chromatography
UHPSFE:	ultrahigh performance supercritical fluid extraction
VLE	Vapor liquid equilibrium

Contents

1	Intro	duction	17	
	1.1	Plant bioactive compounds	17	
	1.2	Lipids	17	
	1.3	Fatty acids and associated lipids		
	1.4	Essential oils	19	
	1.5	Carotenoids		
	1.6	Phenolic compounds		
2	Aims	s of this work	23	
3	Extra	action techniques for bioactive compounds	25	
	3.1	Conventional solid-liquid extraction	25	
	3.2	Pressurized liquid extraction		
	3.3	Supercritical fluid extraction		
	3.4	Carbon dioxide expanded liquid extraction		
4	Extra	action theory for solid samples		
	4.1	Extraction steps		
	4.2	The extraction solvent – solubility		
	4.3	Mass transfer		
5	Supe	Supercritical fluid chromatography applied to lipid analysis		
	5.1	Method development in SFC	47	
		5.1.1 Sample/analyte properties	47	
		5.1.2 Choice of mobile phase		
		5.1.3 Additives		
		5.1.4 Stationary phase		
		5.1.5 Injection solvent		
		5.1.6 Optimization of chromatographic conditions 5.1.7 Detector		
6	Cher	Chemometric tools and data processing		
	6.1	Design of experiments		
	6.2	Data processing		

	6.3	Data exploration	58
7	Societ	al impacts of this research	61
	7.1	Lingonberry as a food supplement	61
	7.2	Oil and oil-soluble compounds	63
	7.3	Utilisation of food waste in a circular economy	64
8	Conclusions and future work		
Ackno	owledg	ements	69
Refere	ences		70

1 Introduction

1.1 Plant bioactive compounds

The bioactive compounds found in plants are heterogeneous phytochemicals produced in plants as secondary metabolites. Secondary metabolites do not have any obvious function in the growth and development of the plant, however, some of them act as defensive agents against pathogenic microorganisms and insects¹. These compounds are found at various concentrations in different plants, including fruits, vegetables and whole grains². They are called "bioactive" as they influence the physiological or cellular activity of living organisms³. It has been suggested that these compounds can modulate the metabolic processes of biological systems leading to better health⁴. Their beneficial effects on health and disease prevention are the result of their antioxidant activity, inhibition or induction of enzymes, inhibition of receptor activities, and induction and inhibition of gene expression⁵. It has also been suggested that bioactive compounds can reduce the risk of various diseases, such as cardiovascular diseases and cancer, as well as other disorders, such Alzheimer's disease⁶.

Bioactive compounds in plants include many classes of chemical structures. Essential oils, phenolic compounds, phytosterols, carotenoids and tocopherols, and organosulfur compounds are examples of bioactive compounds found in plants⁷. These compounds vary enormously in their physicochemical properties, for example, polarity and stability. Therefore, there is a need to develop and optimize analytical techniques and methods taking into account the nature of the target compound. In the present work, qualitative and quantitative extraction and separation methods were investigated and developed to study essential oils in *Boswellia sacra* resin, phenolic compounds and carotenoids in sea buckthorn pomace, and lipids in Moringa and other berry seeds.

1.2 Lipids

The term lipids is derived from the Greek *lipos*, meaning fat⁸. Lipids are defined as any group of organic compounds that are insoluble in water, but soluble in organic solvents, such as ethers and alcohols⁹. These chemical properties cover a broad

range of molecules, such as fatty acids, phospholipids, sterols, sphingolipids, terpenes and others¹⁰. The classification of lipids is difficult because of their diversity, and several systems have been constructed. Furthermore, lipids have been broadly subdivided into "simple" and "complex" lipids; simple lipids being those yielding at most two types of product upon hydrolysis (e.g., fatty acids, sterols and acylglycerols), and complex lipids (e.g., glycerophospholipids and glycosphingolipids) yielding three or more products upon hydrolysis¹⁰. The work presented in this thesis concerns lipids from plants that have structural and functional roles in the human metabolism.

1.3 Fatty acids and associated lipids

Fatty acids are mono-carboxylic acids with long branched or unbranched aliphatic carbon chains, which may be saturated or unsaturated⁹. A 2-carbon acetyl is the most common precursor in fatty acid synthesis. For this reason, fatty acids with even numbers of carbon atoms are more common than those with odd numbers. The length of the carbon chain and the degree and position of unsaturation are the main characteristics distinguishing between fatty acid species¹¹. Carbon chain lengths between 2 and 36 carbon atoms are common in nature; the most abundant having 12 or 22 carbons¹². In the human body, fatty acids are mainly synthesized in the liver and adipose tissues¹³. The human body has the ability to synthesize saturated and monounsaturated fatty acids. However, we lack the enzymes necessary to introduce double bonds beyond carbon 9 in the chain¹⁴. Thus, linoleic acid and linolenic acid (C18:2 and C18:3, respectively) cannot be synthesized in the body. In contrast, plants and algae contain the enzyme desaturase allowing the formation C12 and C15 and, as a result, linoleic acid and linolenic acid are two of the most predominant fatty acids found in plants¹⁵. Fatty acids are major components of other classes of lipid such as glycerolipids, phospholipids, sphingolipids and glycolipids. These types of lipids are the main components of the cell membrane in plants. Studies have shown that foods rich in ω -3 polyunsaturated fatty acids have beneficial effects on inflammation¹⁶ and non-alcoholic fatty liver disease¹⁷. There are many challenges in lipid analysis due to the diversity of lipid structure, and it is important to choose an appropriate solvent. Matrix effects must also be considered as these lipids are the main components of the cell membrane, and polar lipids may interact strongly with other cellular components through hydrogen bonds, ionic bonds or covalent bonds¹⁸. In addition, recent improvements in supercritical fluid chromatography have increased the potential to develop separation methods for lipid profiling with high resolution and selectivity.

Two sample preparation methods were investigated and optimized in the present work for the extraction of fatty acids and their derivative lipid classes. **Paper III** describes a study on the use of ultrahigh-pressure (up to 80 MPa) supercritical fluid

CO₂ (ScCO₂) for the extraction of oil from Moringa seeds. The paper discusses how the amount and composition of the oil extracted are affected by the density of the ScCO₂ and how the presence of a modifier can enhance the solubility of more polar lipids. **Paper IV** describes a method of extracting oil from various berry seeds using a combination of CO₂ expanded ethanol (CXE) and sonication to enhance the mass transfer properties and oil recovery. The method was optimized and applied to extract lipids from different berry seeds. **Paper II** describes the development of a chromatographic method for global lipid profiling using ultrahigh performance supercritical fluid chromatography (UHPSFC) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS). Seven stationary phases were screened to find the best one for the separation and detection of the lipids.

1.4 Essential oils

Essential oils are volatile natural compounds that give the plant its characteristic odour and flavour¹⁹. They are obtained from certain plants using steam or mechanical processes such as pressing techniques²⁰. Characteristic components of essential oils are terpenes, which differ from fatty oils in that they do not contain glycerides of fatty acids. Some essential oils contain saturated and unsaturated aliphatic, aromatic, terpene, sesquiterpene, mono- and bicyclic hydrocarbons, and their oxygen derivatives. Terpenes and terpenoids are the main constituents of essential oils, isoprene being the building block of these constituents²¹. Many studies have been conducted to evaluate the bioactivity of essential oils, and it has been suggested that essential oils have antimicrobial, anti-aging, antioxidant and anti-inflammatory properties²². Essential oils can be extracted using solid-liquid extraction (SLE), however, slow mass transfer is one of the main drawbacks, together with the requirement of large volumes of solvents. Mass transfer can be improved by raising the temperature, but this can cause the loss of volatile components. Supercritical fluid extraction (SFE) at moderate temperatures is a good alternative for essential oil extraction, however, expansion of the gas at the outlet might also cause the loss of the target compound. Therefore, the solubility and extraction kinetics of two constituents of essential oils, α -pinene and cis-verbenol (as a model) from *Boswellia sacra* resin were studied using CXE (Paper I). Since no commercial instrument was available for gas-expanded liquid extraction, the possibility of using a SFE instrument to perform CXE extraction was investigated. Extract collection, theoretical solubility parameters and a comparison of the extraction rates of CXE with conventional techniques are described in Paper I.

1.5 Carotenoids

Carotenoids are a diverse group of natural pigment compounds that have important biological roles and functions in the animal and plant kingdoms²³. They are responsible for yellow and orange colours, and are synthesized in plants by photosynthesis from a building block called the isoprene unit²⁴. They can, therefore, not be synthesized in animals, and are instead obtained from the diet. Carotenoids play a vital role in health, by preventing the short-wavelength radiation in the eye and also regulating and modifying some of the physical properties of biomembranes²³. Carotenoids also have radical scavenging properties, and are therefore considered to be antioxidants. The analysis of carotenoids is not an easy task due to the many challenges related to matrix effects, solubility, stability and identifying suitable separation and detection techniques²⁵. These challenges will be discussed in detail in Chapters 2 and 3. In the study described in **Paper V**, β -carotene was extracted from sea buckthorn pomace using CO₂ expanded liquid (CXL) coupled to in-line UV-vis absorption spectrophotometry detection. The solubility and extraction kinetics of the carotenoids were investigated using continuous CXLE.

1.6 Phenolic compounds

Phenolic compounds are a class of chemical compounds consisting of a hydroxyl group (-OH) directly bound to an aromatic hydrocarbon group. They are secondary metabolites in plants and play a role in protection, as well as contributing to the colour and sensory characteristics of fruits and vegetables²⁶. They show a considerable diversity in structure, ranging from simple molecules such as gallic acids, to polymeric molecules such as condensed tannins²⁷. Phenolics are classified as phenolic acids, flavonoids, stilbenes and lignin²⁸. Over 8000 different structures have been reported in the flavonoid family alone²⁹. Phenolic compounds are associated with various health benefits, presumably via anti-allergenic³⁰, antimicrobial³¹ and antioxidant³² mechanisms. The extraction of phenolic compounds for qualitative and quantitative analysis is a true challenge due to matrix effects and the instability of the analytes. In general, antioxidants including phenolic compounds differ in their heat stability. Many of the antioxidants that exhibit bioactivity at ambient temperatures are rapidly broken down and lose their effectiveness when exposed to elevated temperatures³³. Phenolic compounds are normally extracted using conventional techniques such as SLE, or techniques operated at high temperatures such as pressurized liquid extraction (PLE)³⁴. Slow mass transfer and the risk of degradation are the main drawbacks of currently available extraction techniques for phenolic compounds. Therefore, the possibility of using gasexpanded liquid (GXL) extraction at low temperatures to improve mass transfer and

extraction rate was investigated (**Paper V**). Figure 1 presents the chemical structures of some bioactive compounds.



Figure 1. Structures of some of the compounds investigated in the present work.

2 Aims of this work

The studies presented in this thesis were intended to address the following research questions:

- How efficient in terms of selectivity and recovery is CO₂ expanded liquid extraction compared to conventional extraction techniques?
- Can CXLE offer high recovery and a high extraction rate at low temperatures for unstable bioactive compounds?
- How are the selectivity and extractability of supercritical fluid CO₂ affected by ultrahigh pressure?
- How can a stationary phase with high chromatographic efficiency and selectivity be identified for lipid profiling using supercritical fluid chromatography?
- What effects does the intake of lingonberries have on plasma metabolites and their profiles, and thus on health?

3 Extraction techniques for bioactive compounds

Bioactive compounds in plants are often synthesized in small quantities, and are either unconjugated, as aglycones, or conjugated with sugars³⁵. Qualitative and quantitative studies of these compounds rely mainly on the use of the correct sampling method and sample preparation technique³⁶. The sample preparation and extraction methods should be carefully chosen bearing in mind the matrix complexity, the type of target analytes, the location of the analytes within the matrix, applications and instruments availability.

The techniques used for the extraction of bioactive compounds from plants have previously been developed from traditional techniques. However, significant advances have been made in extraction techniques in term of selectivity, sustainability, speed and automation.

3.1 Conventional solid-liquid extraction

Conventional SLE is one of the most widely used extraction techniques for bioactive compounds from solid samples. Steeping of a tea bag in hot water is the simplest example of conventional SLE. Maceration, hydro-distillation and Soxhlet are other examples of conventional SLE techniques used to extract bioactive compounds from plants³⁷. Extraction can be performed in batch or continuous flow mode. In ideal SLE, the desired compound(s) in the matrix should have high solubility in the solvent employed, while other compounds tend to remain in the matrix³⁸. The selectivity and efficiency of extraction rely on the extracting power of the solvent, and heating and/or stirring may change the selectivity. In batch mode extraction, the ratio of solvent to sample is important to ensure that at equilibrium (nearly) all the analytes diffuse from the matrix into the solvent³⁹. In general, conventional SLE methods require a large volume of toxic solvents, which means large volumes of waste. In addition, the use of a toxic solvent prevents the application of these methods in the pharmaceutical and food industries. These methods are also rather slow due to slow mass transfer. Moreover, this kind of extraction requires clean-up and solvent evaporation, which prolong the process and increase the cost of extraction. Increasing the temperature could enhance the extraction process by decreasing solvent viscosity, thus improving the mass transfer properties. However, higher temperatures cannot be used in the case of thermally unstable target compounds. High temperatures may also lead to evaporation of the solvent, leading to a change in the solvent-to-sample ratio during the process. Conventional SLE was used as a reference method in the studies described in **Papers III** and **IV**.

3.2 Pressurized liquid extraction

A considerable improvement in extraction technology was achieved in 1996 when Richter et al. developed PLE^{40} . This method is now known by several other names: accelerated fluid extraction, enhanced solvent extraction, and high pressure solvent extraction⁴¹. PLE is based on the use of liquid solvents (aqueous or organic) at high temperatures and elevated pressure to maintain the liquid state of the solvent. Increasing the temperature beyond the atmospheric boiling point at high pressure causes changes in the physicochemical properties of the solvent, such as the dielectric properties (intermolecular interactions that can be established between the solvent and the analytes to be dissolved⁴²) and the viscosity. For example, Luong et al. found that the dielectric constant of water at 200 °C and 10 MPa was equivalent to the dielectric constant of acetonitrile at room temperature⁴³. Also, the viscosity of water at 200 °C is about five times lower than under ambient conditions⁴⁴. PLE was found to have several advantages over conventional SLE techniques, namely shorter extraction times, higher recoveries and less solvent is required⁴⁵. Sample waste and thus costs are also reduced as sample extraction and clean-up can be performed in a single step⁴⁶. A wide range of organic solvents (from water to hexane) have been used in PLE, depending on the polarity of the target compound. In recent years, pressurized hot water (PHW) has been widely used to extract bioactive compounds⁴⁷. The changes in the dielectric constant, viscosity and solubility parameters of PHW in the subcritical range (Figure 2B) make it an alternative solvent to toxic organic solvents. PHW has been used to extract betulin, alkaloids and phenolic compounds^{48,49}.

Although PHW is used widely for the extraction of bioactive compounds, it still suffers from some disadvantages related to degradation due to the high temperature and the loss of bioactivity caused by the oxidation of the functional groups⁵⁰. Another disadvantage of PHW extraction is that when the extracts cool to ambient temperature there is a risk of precipitation due to changes in the solvent properties. An organic solvent is therefore added to the water (5-10%) to avoid precipitation. The PHW extraction of total phenolic compounds from lingonberry samples is described in **Paper VI**.



Figure 2. Phase diagrams of water (A) and CO₂ (B).

3.3 Supercritical fluid extraction

Supercritical fluids are substances that exist as a single phase above their critical point of pressure and temperature. The critical point or critical state is defined as the condition where vapour and liquid are indistinguishable and no phase boundaries exist. For example, the supercritical condition for water is at 22.7 MPa and 374 °C (Figure 2A) and CO₂ is found at 7.3 MPa and 31 °C (Figure 2B). Supercritical fluids are attractive because of their low viscosity (gas-like) and high density (liquid-like), which enhance their mass transfer and solubility properties, respectively⁵¹. Table 1 gives the general properties of some supercritical fluids. Supercritical propanebutane⁵², water⁵³, ammonia⁵⁴ and CO₂⁵⁵ have been used in analytical chemistry. The most commonly employed supercritical fluid is CO₂, because of its low critical temperature and pressure (31 °C and 7.3 MPa), inertness, purity, non-toxicity and availability. In addition, the ScCO₂ solvation strength can be tuned by altering the temperature and pressure. Another advantage of CO_2 is that it is gaseous at room temperature and ambient pressure, which makes analyte recovery relatively simple and inexpensive, and solvent-free extracts can be obtained. Also, the fact that ScCO₂ can be run at low temperatures using a non-oxidizing medium is important for sample preparation of food, biological and natural products, which allows the extraction of thermally labile or easily oxidized compounds with minimum degradation⁵⁵. Due to its low dielectric constant and a dipole moment close to zero, neat $ScCO_2$ has solubilizing properties similar to those of *n*-hexane and *n*-heptane. However, it has a quadrupole moment due to charge separation and its electronic structure, which enables it to act as a Lewis acid and a Lewis base^{56,57}. Although ScCO₂ has a quadrupole moment, it still behaves like a nonpolar solvent, which

limits it application for the extraction of more polar compounds. To overcome this limitation, a polar modifier or (co-solvent) is usually added to tune the polarity and enhance the solvating power. Methanol, ethanol and ethyl acetate are examples of organic solvents that are added at relatively small levels (1–20 vol%) to ScCO₂ to expand its extraction range to include more polar analytes.

Table 1. Physical properties (density, diffusion and viscosity) of gaseous, superchitical and riquid states.				
State	Density (g/cm ³)	Diffusion (cm ² /s)	Viscosity (g/cm•s)	
Gas	10 ⁻³	10 ⁻¹	10-4	
Supercritical	10 ⁻¹ - 1	10 ⁻⁴ - 10 ⁻³	10 ⁻⁴ - 10 ⁻³	
Liquid	1	<10 ⁻⁵	10 ⁻²	

Table 1. Physical properties (density, diffusion and viscosity) of gaseous, supercritical and liquid states⁵¹.

The principal components of an SFE instrument are a CO₂ source, a high-pressure pump, a heating chamber in which the extraction vessel is placed, and a restrictor to maintain the desired pressure⁵⁸ (Figure 3). Several commercial SFE instruments are available on the market, but equipment can also be assembled in the lab. A CO₂ cylinder can be equipped with a dip-tube to ensure that the CO₂ leaving the cylinder enters the pump in liquid form. Liquid CO₂ has a higher density than the gas, which stabilizes the flow from the pump. Purity of the liquid CO₂ is essential to avoid contamination and prevent ice formation resulting from the presence of water. Both syringe and reciprocating piston pumps have been used in SFE. The pump head should be coated with a cooling exchanger to cool the liquid and increase its density. A syringe pump provides a more stable and smooth flow due to the absence of pulsation. This enables extraction at high flow rates⁵⁹. The only drawback of syringe pumps is the limited volume of the syringe, and this may cause interruption of extraction for refilling. Reciprocating pumps are less expensive than syringe pumps, but their volumetric delivery is not as accurate, especially at low flow rates, and their maximum flow is limited. Furthermore, reciprocating pumps build up pressure upstream, and a long time is often required to reach the desired pressure, particularly at low flow rates. In syringe pumps the situation is the opposite (downstream) and the liquid CO_2 is pumped at the set pressure. In the study described in **Paper I**, the instrument was equipped with a reciprocating pump (Waters), while in-house setups equipped with a syringe pump (ISCO) were used in the other studies (Papers III and V).



Figure 3. Schematic diagram of the commercial SFE instrument used⁶⁰.

The heating chamber in the SFE system controls the temperature of the extraction process. The chamber is equipped with a circulating fan to distribute the heat evenly. Two important aspects of the heating chamber should be considered. The first is a pre-heating loop (1-2 m long) before the extraction vessel inlet to ensure that the fluid reaching the vessel is at the desired temperature. The second is that the vessel should be oriented vertically with the inlet at the bottom. This will enhance sample agitation and reduce channelling effects. The restrictor or back-pressure regulator (BPR) controls the flow of the fluid to maintain a high pressure in the extraction vessel. The most common techniques used for pressure restrictors in SFE are a simple capillary restrictor or a mechanical needle regulator⁶¹. The length and diameter of the capillary tube determine the flow resistance that can be built up and thus the back-pressure. A capillary tube restrictor is simple and inexpensive, but it is difficult to achieve reproducibility. It also suffers from clogging caused by extract precipitation or ice formation due to rapid expansion of CO₂. In the study described in Paper III a stainless-steel tube with a diameter of 0.006 inches connected to a needle valve was used to control the extraction flow rate, while the syringe pump was run at constant pressure. The restrictor tube was placed inside a heating chamber to avoid blockage due to extract precipitation or ice formation. Mechanical restrictors, on the other hand, consist of mechanically adjustable valves (needle valves) and a seat. The gap between the needle and the seat controls the pressure and flow rate. A mechanical restrictor can be controlled manually by turning the valve clockwise or anticlockwise, or electronically by connection to a density sensor programmed to measure the solvent density, which then controls the gap between the needle and the seat⁶². The BPR is connected to a thermocouple to maintain a certain temperature, to avoid ice formation and extract precipitation.

Makeup solvent is sometimes added after the extraction vessel to increase the solubility of the extract and to avoid precipitation. The makeup solvent can be added before or after the BPR depending on the percentage of co-solvent used. It is preferable to add the makeup solvent before the BPR but this is not always possible as the makeup pump may not be able to handle the extraction pressure. The commercial SFE system (MV-10 ASFE system, Waters) is shown in Figure 3 is equipped with a transfer line and heat exchanger, the role of which is to slow down the expansion of the CO₂ as it is depressurized. Using this set-up, the recovery of α -pinene was increased when chilled make-up solvent was added after the transfer line. This shows that the heat from the transfer line can influence the volatility of the extract during the collection stage.

3.4 Carbon dioxide expanded liquid extraction

In SFE, a modifier or organic solvent can be added to the supercritical fluid at small percentages (1-20 vol%) to increase the polarity of the solvent. In gas GXLs, the concept is the opposite; up to 50% compressed liquid gas (usually CO_2) is added to an organic solvent. This concept has been investigated in liquid chromatography as enhanced fluidity, for example, by Olesik and her group⁶³. GXLs have been used in including chromatographic applications separation. fine-particle manv precipitation, polymer processing, and as reaction media for catalytic reactions⁶⁴. When compressed gas is dissolved in an organic solvent, this causes a 2-6 fold volumetric expansion of the liquid^{64,65} depending on the ratio between the liquid CO_2 and the organic solvent, the temperature and the pressure. Expansion is the result of changes in physicochemical properties such as the viscosity, dielectric constant and density⁴². GXLs are found at lower temperatures (25-60 °C) and moderate pressures (6-10 MPa), compared to ScCO₂. Figure 4A shows the mixing of liquid CO_2 (0.34 molar fraction) and ethanol in a microchip channel at ambient temperature and under 6 MPa pressure.

GXLs have unique combined gas and liquid properties. The addition of CO_2 to the organic liquid decreases the viscosity and interfacial tension of the liquid, thus improving the diffusivity and mass transfer properties⁶⁶. The polarizability (i.e., the ability of the solvent to induce electrostatic interactions with a dissolved analyte) of GXLs has been studied for different solvents⁶⁷. In general, the polarizability decreases with increasing fraction of CO_2 and increasing temperature. In contrast to ScCO₂, GXLs have the ability to solubilize analytes with a wide range of polarities. GXLs have been shown to be effective solvents for oil recovery, gas recrystallization⁶⁸, as the mobile phase in high performance liquid chromatography (HPLC)⁶⁹, and as media for catalytic reactions⁷⁰ and particle formation⁷¹.



Figure 4. (A) Microchip flow cell illustrating the mixing of CO₂ (0.34 molar fraction) in ethanol at 6 MPa and room temperature (with permission from Martin Andersson, Uppsala University). (B) phase diagram of a binary system of CO₂ and ethanol at 40, 60 and 80 $^{\circ}C^{42}$.

It is important to consider the vapour–liquid equilibrium (VLE) of a binary system, particularly when CO_2 is one of its components. The VLE describes the distribution of the molecules of a pure component or a mixture of components between the vapour and liquid phases. The resulting mixture of CO_2 and organic solvent consists of a dense liquid phase containing dissolved CO_2 , and a vapour phase consists of CO_2 and organic liquid⁴². A single homogeneous phase can be obtained when pressure is applied to merge the liquid and vapour phases. Increasing the temperature has the opposite effect. Figure 4B shows the phase diagram of the binary system of CO_2 and ethanol at 40, 60 and 80 °C. The temperature, pressure and molar fraction of CO_2 determine the phase boundary (where bubbles of gas start to form).

CXLE can be performed in the two-phase region or in one single-phase. The feature of a two-phase region is that the composition of the liquid phase does not change when changing the molar fraction of CO_2 at fixed temperature and pressure⁴². Two-phase CXL has been used as a solvent for chemical synthesis and polymerization⁶⁵. When two-phase CXL is used for extraction, pumping from the liquid phase need be considered in the design of the set-up. Knowledge of the VLE behaviour of the system is important to optimize the design and operation of an extraction process. This is because the density of the solvent in a two-phase system relies on the liquid density, while in one phase the density of the solvent is governed by the temperature, pressure and molar fraction of CO_2^{42} . Therefore, the VLE was considered when choosing the range of CXLE parameters in the studies described in **Papers I, IV** and **V**.

The properties of GXLs in terms of solubility, selectivity and extraction rate, as extraction solvents in single-phase solvents have not yet been fully addressed. The extraction kinetics of two components of an essential oil from *Boswellia sacra* resin were investigated using CXE (**Paper I**), while the study described in **Paper IV** was performed to investigate the combination of CXLE and sonication to enhance the mass transfer, and thus the extraction rate, of lipids from various berry seeds. The solubility, extraction kinetics and degradation of quercetin and β -carotene from sea buckthorn pomace were investigated using CXLE combined with in-line UV-vis detection (**Paper V**), using the in-house CXLE system shown in Figure 5.



Figure 5. Schematic diagram of the in-house CXLE system. MFM: mass flow meter. CCD: chargecoupled device, BRP: back-pressure regulator.

It can also be seen from Figure 4A that mixing CO_2 and ethanol does not occur fast and it take time to form one single phase. It was therefore investigated whether the flow after mixing at the T-junction reaches the extraction vessel as one phase or not. The UV-vis response provides an indication of whether one or two phases have passed the detector. One phase will give a stable, flat signal, while two phases will lead to a fluctuating signal. In the study presented in Paper V a simple set-up, as shown in Figure 6, was built to determine the required distance between the Tjunction and the extraction vessel for a single-phase solvent. A stainless-steel tube with an OD of 1/16" and ID of 0.25 mm (the same dimensions as the tube used in the commercial system (Paper I) and the in-house system (Paper V)) was used for the part labelled "d" in Figure 6. The most crucial conditions for the GXLs used in extraction in these studies are high temperature and high molar fraction of CO_2 as the solubility of CO_2 decreases with temperature, and the probability of division into two phases is high.



Figure 6: The set-up used to investigate the distance required to obtain a single-phase using the inline UV-vis detector.

The results showed that at 80 °C and 12 MPa, with a 0.5 molar fraction of CO_2 in ethanol, and a total flow mass of 2.0 g/min, the tube length required between the T-junction and the detector to give a stable signal was between 80 and 90 cm (unpublished data). Hence, using a coil of tubing about 1 m long before the extraction vessel is adequate to ensure the mixing of CO_2 in ethanol, resulting in single-phase when reaching the vessel. Table 2 summarizes the properties of the extraction techniques discussed above.

Technique	SLE	PLE	SFE	CXLE
Temperature (°C)	25-60	50-200	40-80	25-70
Pressure (MPa)	Ambient	5-10	10-80	5-10
Viscosity	High	Medium	Low	Low to medium
Mass transfer	Slow	Fast	Fast	Fast
Degradation risk	Low to high	High	Low	Low to medium
Target analytes	Polar to nonpolar	Polar to nonpolar	Nonpolar	Polar to nonpolar

Table 2. Summary of the properties of the extraction techniques SLE, PLE, SFE and CXLE
4 Extraction theory for solid samples

4.1 Extraction steps

The qualitative and quantitative analysis of bioactive compounds is influenced by the choice of extraction technique and operation conditions. To understand the fundamentals of any solid–liquid extraction technique it is necessary to know the general kinetics and steps involved in the extraction. In theory, the analytes are attached to the sample matrix with different strengths and at different positions⁷², which may influence the extraction process and kinetics. The analytes may be available on the surface of the particle, or loosely bound to the matrix, as indicated by positions 1 and 2 in Figure 7. Some analytes may be dissolved or loosely integrated in the micro/nanopores of the matrix (3 and 4 in Figure 7), while others may be strongly incorporated into and/or chemically bound deep inside the matrix particles, as in position 5. These strongly and deeply bound analytes are the most challenging to extract since force is required to break the chemical bonds and cause them to diffuse into the bulk solvent.



Figure 7. A schematic illustration of analytes differently bound to/incorporated in a matrix: (1) dissolved in the bulk solution, (2) adsorbed on the surface of the matrix, (3) dissolved in the pore solvent and/or adsorbed on the pore surface, (4) dissolved in micro/nanopores or adsorbed on the walls of the micro/nanopores, and (5) chemically bound to the matrix. (Adapted from Ref⁷².)

Pawliszyn described the basic steps involved in extracting analytes from a solid sample matrix into the bulk solvent⁷³. Firstly, the analytes desorb from their binding

site and diffuse through the organic part of the matrix. They then reach the interface between the matrix and the solvent in the micro/nanopores. When they reach the organic phase, they dissolve and diffuse through these micro/nanopores to reach the Nernst diffusion layer. After this step, the analytes diffuse into the bulk solvent. Desorption/diffusion of the analytes from the sample matrix into the solvent, and dissolution/partitioning of the analytes from the Nernst diffusion layer into the bulk solvent are the main steps determining the extraction rate. Hawthorne et al. investigated the change in extraction rate in SFE when varying the flow rate⁷⁴. They found that plotting the amount of fat extracted from potato chips using $ScCO_2$ (34 MPa and 60 °C) as a function of flow rate gave almost straight lines until extraction was completed (Figure 8A)⁷⁴. When the amount extracted is linearly dependent on flow rate this indicates that extraction is mainly limited by the solubility/partitioning step. When the amount extracted is plotted versus solvent volume at different flow rates (Figure 8B), the data coincide, indicating that the same amount of fat is extracted per unit volume of solvent, regardless of the flow rate. This also indicates that the analytes are readily available at the interface between the matrix and solvent.



Figure 8. An example of a case where extraction is limited by the solubility/partitioning of fat from potato chips using SFE at 34 MPa and 60 $^{\circ}C^{74}$. (A) The extraction profile, shown as amount vs. time, is linear for all flow rates. (B) Plotting the amount extracted vs. solvent volume shows that increasing the solvent flow increases the amount extracted (reprinted with permission).

In contrast, little or no change is seen in the extraction rate with varying flow rate in extractions that are controlled by desorption/diffusion. The amount extracted changes gradually over time regardless of the flow rate. This is because the extraction rate depends on the diffusion of the analytes from the core of the matrix to the surface. In the present work (**Paper I**), the extraction of cis-verbenol from *Boswellia sacra* using CXE at different flow rates showed that extraction is controlled primarily by solubility/partitioning during the early stages of extraction (i.e., a linear relationship) (Figure 9). The extraction rate then decreased at every flow rate. This can be explained in two ways. The first is that all the analytes available on the surface have been extracted, and the desorption/diffusion of those bound to the matrix determines the extraction rate. The second is that since the partitioning of an analyte depends on the distribution constant, and since only a small amount is left in the matrix after extraction of the analytes available on the surface, the solubility, rather than diffusion, may still play a role in controlling the extraction rate.



Figure 9. An example of a case in which extraction is limited initially by solubility/partitioning (5 min) and then by desorption/diffusion when cis-verbenol is extracted from *Boswellia sacra* resin using CXLE⁶⁰. (A) Extraction is initially limited by solubility/partitioning but as there were too few sampling points, this is only seen at 5 min. After this time, extraction is limited by desorption/diffusion, since doubling the flow rate does not double the amount extracted. (B) Extracted amount vs. solvent volume plot shows different extracted amounts were obtained by unit volume at different floe rates.

4.2 The extraction solvent – solubility

Solubility is defined as "the analytical composition of a saturated solution, expressed in terms of the proportion of a designated solute in a designated solvent"⁷⁵. Expressed more simply, solubility is the amount of an analyte that can be dissolved in a certain amount of solvent or solution at a specific temperature and pressure. The strength of the intermolecular forces between solutes and solvents determines the solubility of a given solute in a given solvent⁷⁶. Solutes successfully dissolve in solvents when the solute–solvent bonds are stronger than either the solute–solute bonds or the solvent–solvent bonds. High solubility may lead to a high extraction rate and thus efficient extraction. However, other factors can improve the solubility of the target analyte apart from the choice of the extraction solvent.

The general rule of "like dissolves like" is usually applied when choosing a solvent. Using the appropriate solvent ensures a high recovery in a short period of time, which means less solvent consumption. Different approaches can be used to predict or measure the solubility of an analyte in a solvent or mixture of solvent at defined temperature, pressure and pH. Some of these approaches rely on theoretical calculations (of intermolecular forces) while others require experimental measurements. The structure of the target analyte is used in theoretical approaches to calculate group contribution, in order to predict its solubility in the intended solvent. These methods involve the calculation of the cohesive energy density of the solvent, that is, the energy required to break intra- and intermolecular forces⁷⁷. Hansen solubility parameters (HSPs) are commonly used to predict solubility based on the cohesive energy density. Molecular interactions are categorized into three groups: dispersive interactions (δ_d), polar interactions (δ_p), and hydrogen bonding (δ_h). The prediction of the suitable solvent(s) based on finding solvents that has HSPs very close to HSPs of the analyte. HSPs have been used to find a suitable extraction solvent for lipids⁷⁸, phlorotannins⁷⁹ and other groups of compounds.

$$\delta_t^2 = \delta_d^2 + \delta_p^2 + \delta_h^2$$

The solubility of an analyte can be measured experimentally using static or dynamic methods. The complexity of the experimental set-up depends on the operating conditions, for example, high pressure, which is the case here. In the static method, equilibrium is achieved between the analyte and the solvent before sampling. Equilibrium can be achieved by recirculation of the solvent, agitation, or leaving the solvent and solute to equilibrate for some time. Cunico et al. measured the solubility of curcumin in CXE using circulation to achieve equilibrium and online sampling connected to supercritical fluid chromatography (SFC)⁸⁰. In the dynamic method, the analyte is in contact with the solvent during the flow of the solvent inside the extraction vessel. The flow rate plays a role in controlling the residence time of the solvent, a low flow rate is very important to increase the residence time of the solvent, to ensure that the system reaches equilibrium. The amount of analyte in the collected extract is measured, and the solubility can be expressed in terms of mass fraction or mole fraction.

In the present work, HSPs were used to match the solvents ScCO₂, CXE and pure ethanol to the analytes α -pinene and cis-verbenol (**Paper I**). However, HSPs are affected by the temperature and pressure of the solvent. Generally, an increase in pressure at constant temperature will increase the total solubility parameter due to the increase in the solvent density, while an increase in the temperature at constant pressure will decrease the total solubility parameter⁸¹. Table 3 gives the solubility parameters for common solvents at different temperatures and with CO₂ addition. The addition of a 0.31 molar fraction of CO₂ to ethanol at 9.3 MPa and 40 °C decreased the values of the HSPs of ethanol (δ_d , δ_p and δ_h) from (15.8, 8.8, 19.4) to (8.5, 5.0, 11.1). This indicates that CXE is suitable for the extraction of more nonpolar components such as cis-verbenol, which has δ_d , δ_p and δ_h values of 16.8, 3.1 and 8.4, respectively.

Another example of the change in the HSPs in a solvent is PHW, which was used in the present work to extract phenolic compounds at 99 °C (**Paper VI**). Hydrogen bonding (δ_h) is the main effective HSP of water, which is also the parameter most affected by temperature (Table 3)⁸². Quercetin, for example, has HSP values of 23.0, 15.8 and 25.0 for δ_d , δ_p and δ_h , respectively, and the value of δ_h for water decreases from 42.3 to 36.3 when the temperature is increased from 25 °C to 99 °C. The value of δ_h for ScCO₂ is also affected by the pressure and temperature. Increasing the pressure increases the density of the solvent, which causes more molecules to interact⁴². King et al. estimated the value of δ_h for ScCO₂ at 40 °C and different pressures to be: 5.57 at 10 MPa, 7.92 at 30 MPa, 8.66 at 50 MPa, and 9.12 at 70 MPa⁸³.

(o _P), and hydrogen bonds (o _H) for selected solvents under different conditions ^{00,01}					
Solvent	Conditions	δ _d	δ _p	δ _h	
Water	0.1 MPa/25 °C	15.5	16.0	42.3	
Water	5 MPa/99 °C	13.4	14.2	36.3	
Ethanol	0.1 MPa/25 °C	15.8	8.8	19.4	
ScCO ₂	10 MPa/ 40 °C	16.3	4.4	5.1	
ScCO ₂	25 MPa/ 40°C	17.8	5.2	7.0	
CXLE*	9.3 MPa/ 40 °C	8.5	5.0	11.1	

Table 3. Hansen solubility parameters consisting of dispersive interactions (δ_D), polar interactions (δ_P), and hydrogen bonds (δ_H) for selected solvents under different conditions^{60,81}

* CXLE: ethanol with a 0.31 molar fraction of CO2

The effects of changes in HSPs were also observed in the present work when ScCO₂ was used to extract oil from Moringa seeds (Paper III). Increasing the pressure from 40 to 80 MPa at 57 °C increased the density of the ScCO₂ fluid from 0.90 to 1.03 g/ml. Increasing the density increases the solubility of oil components in ScCO₂ due to an increase in solvating power. About 400 mg of oil was extracted from 1 g seeds after flushing the vessel with 30 g of CO₂ with a density of 1.03 g/ml, while flushing with about 40 g CO₂ with a of density is 0.90 g/ml was sufficient to extract about 278 mg oil (Figure 10). Increasing the density of ScCO₂ also enhances the extraction of polyunsaturated fatty acids, and some polar lipid classes were also extracted, namely PC, PE and LPC. It has been suggested that increasing the pressure increases the dipole moment of ScCO₂, thus increasing its electron donating and accepting capacity, enabling it to react with polar head groups⁸³. Thus, extraction at a lower density, 0.90 g/ml, is more selective for neutral lipids (sterol esters, tri-, di- and monoacylglycerol) and shorter-chain fatty acids. While extraction at higher density is more selective to polar lipids and longer chain fatty acids with higher degree of unsaturation.



Figure 10. Extracted amount of oil from Moringa seeds vs. the amount of solvent using SFE at 40 MPa and 80 MPa and a constant temperature of 57 °C (from Paper III).

When extraction is performed in batch mode, solubility is the more limiting step. In batch mode, extraction reaches equilibrium, which reduces the extraction rate. This problem can be solved by using a high solvent:sample ratio and replacing the extraction solvent with fresh solvent⁸⁴. A high solvent:sample ratio increases the concentration gradient of the analytes between the solid and the solvent, and this is the driving force for mass transfer⁸⁵. Norshazila et al. found that the amount of carotenoids extracted increased two-fold when the solvent:sample ratio was increased from 50:1 to 150:1⁸⁶.

The effect of the solvent:sample ratio was investigated in the present work in the extraction of total lipids from berry seeds using batch CXE extraction (**Paper IV**). The total extracted lipids, determined gravimetrically, increased from 15 to 24 weight % when the solvent:sample ratio was increased from 5:1 to 20:1 (w/w). This indicates that equilibrium was not reached. Hence, replacing the solvent with fresh solvent (repeated extraction cycles) was investigated. The total extracted amount increased significantly, to about 323 mg/g seed sample in the second extraction cycle.

4.3 Mass transfer

Mass transfer plays an important role in the kinetics of extraction, and is defined according to IUPAC as: "The spontaneous (irreversible) process of transfer of mass across non-homogeneous fields. The driving force can be the difference in concentration (in liquids) or partial pressure (in gases) of the component. In fluids, mass transfer may be enhanced by turbulent flow"⁸⁷. Diffusion is the slowest and

rate-limiting form of mass transfer⁸⁸. Diffusion may be self-diffusion (D_i), where a molecule diffuses in the absence of a chemical potential (homogeneous medium), or binary diffusion (D_{12}), which describes the diffusion of a molecule in a solvent in the presence of a chemical potential such as a concentration gradient⁴². Fick's first law describes the flux in terms of a concentration gradient:

$$J = -D_{12}\frac{\partial C}{\partial x}$$

where the flux J [cm⁻² s⁻¹] is proportional to the binary diffusion coefficient (D_{12}) [cm²/s] and the concentration gradient, $\frac{\partial c}{\partial x}$ [cm⁻³ cm⁻¹]. The negative sign indicates that J is positive when the movement is down the gradient. Fick's second law of diffusion describes the rate of change in concentration with time in terms of the concentration gradient⁴².

$$\frac{\partial C}{\partial t} = D_{12} \frac{\partial C}{\partial x^2}$$

In extraction, two main steps are limited by diffusion: diffusion of the analytes from their binding sites through the micro/nanopores to the Nernst diffusion layer, and diffusion of the analytes though the Nernst diffusion layer to the bulk solvent⁷². In the case of diffusion through the Nernst diffusion layer, increasing the flow rate might reduce the thickness of the Nernst diffusion layer, which may enhance mass transfer. However, other parameters can be changed to increase the mass transfer, including temperature, particle size and agitation.

The effect of temperature on extraction has been thoroughly investigated for most common extraction techniques. Increased temperature reduces solvent viscosity, which leads to faster mass transfer. The Stokes–Einstein equation describes the relation between molecular diffusion, temperature and viscosity⁴²:

$$D_i = kT/6\pi\eta r$$

where D_i is the diffusion constant of a molecule in the absence of a chemical potential, k is the Boltzmann constant, T is the temperature, η is the viscosity of the medium and r is the radius of the particle. It can be seen that diffusion is inversely proportional to viscosity. Increasing the temperature decreases the viscosity, leading to increased diffusion. Reducing the viscosity also allows better penetration of matrix particles thus enhancing the extracatibility⁴⁰. It was found in the present work that increasing the temperature of neat ScCO₂ from 40 °C to 57 °C at fixed pressure increased the amount of oil extracted from moringa seeds (**Paper III**). This increase can be attributed to enhancement of the mass transfer properties and the increase in analyte vapour pressure, as discussed in a previous section. However, increasing the

temperature further caused a reduction in the recovery, which may be due to a decrease in the density from 1.07 to 0.987 g/ml when the temperature was increased from 40 to 70 °C at 80 MPa (Figure 11). Roy et al. found that increasing the temperature of ScCO₂ had positive effects on the amount of oil extracted from sunflower seeds at 40 MPa, whereas increasing the temperature at a lower pressure, 20 MPa, led to lower recovery⁸⁹.



Figure 11. Contour plot showing the impact of pressure and temperature on oil recovery (mg/g sample) from Moringa seeds using UHPSFE (Paper III).

The addition of CO₂ to an organic solvent also enhances the mass transfer, as well as allowing the polarizability of the solvent to be tuned. One of the advantages of CXLE is that the viscosity of the solvent is reduced and mass transfer is faster compared to the pure liquid⁹⁰. The extraction rate of α -pinene and cis-verbenol from *Boswellia sacra* using CXE was compared with other extraction methods, SFE and continuous SLE, using Peleg's model (**Paper I**). This model was proposed by Peleg⁹¹ in 1988 to describe the moisture sorption curve, and was later adapted to extraction process. The initial extraction rate using CXLE was faster for both analytes, than with SFE and SLE, as can be seen in Figure 12. Although the mass transfer was faster in SFE than in CXLE, the high solubility/partitioning in CXLE can considerably increase the extraction rate. Remarkable results were found at high flow rate, where CXLE showed a 10-fold higher extraction rate for cis-verbenol than SFE (Figure 12). This suggests that CXLE enables high solubility and fast diffusion rates and can be run at high flow rates.



Figure 12. Comparison of the extraction rate of cis-verbenol from *Boswellia sacra* using three different extraction techniques: CXLE, SFE and SLE at flow rate of 3 ml/min⁶⁰.

Sonication has been used in SLE to enhance recovery and speed up the process. Ultrasound induces convection which can improve mass transfer and eliminate cavitation bubbles⁹². This improves the penetration of the solvent into the matrix, enhancing the diffusion of analytes, particularly those in micro-/nanopores and those chemically bound to the matrix. Paniwnyk et al. found that combining sonication with extraction was more effective at low temperatures⁹³. In the present work, sonication was combined with CXLE for lipid extraction from berry seeds (**Paper IV**). Sonication increased the recovery of lipids by more than 3-fold (Figure 13).

Particle size can also affect mass transfer properties in extraction. Reducing the particle size increases the surface area in contact with the solvent. The influence of this parameter on extraction efficiency has been widely studied. Generally, the smaller the particle size, the higher the amount extracted^{94,95}. This is due not only to the higher surface-to-volume ratio of smaller particles, but also to a reduction in the path along which the analytes must diffuse to reach the bulk phase. When the particle size is very fine (5-50 μ m) internal diffusion can be negligible⁹⁶. In all the studies described here, the solid matrix was crushed to a powder or milled to specific sizes. The powder from sea buckthorn was sieved to the minimum size available in the lab (70 μ m) to eliminate the influence of the diffusion coefficient on the extraction kinetics (**Paper V**). This study is ongoing, and preliminary result are presented.



Figure 13. Impact of sonication on the recovery of selected lipid species (TG: triacylglycerol; FA: fatty acid; PC: phosphatidylcholine and LPC: lysophosphatidylcholine using CO₂ expanded methanol. The obtained peak areas were corrected using corresponding isotopic internal standards. The peak areas obtained with sonication were normalized to the peak areas obtained without sonication (**Paper IV**).

5 Supercritical fluid chromatography applied to lipid analysis

The physicochemical properties of bioactive compounds vary considerably. Therefore, no single separation method has the ability to resolve and detect all of these compounds. It is thus necessary to use a chromatographic separation method to reduce the complexity of the sample extract and facilitate the detection and identification of separate compounds. The choice of separation techniques depends on the kind of target compound and the purpose of the study. For example, high performance liquid chromatography (HPLC) is the most common technique for the analysis of phenolic compounds^{97,98}, while gas chromatography (GC) is a wellknown technique used for essential oil analysis^{99,100}. A simple, rapid, sensitive, selective, low-cost and reliable separation method is always preferable in chromatography. In the present work, HPLC was applied to analyse phenolic compounds from lingonberries (Paper VI), and ultrahigh performance liquid chromatography (UHPLC)/MS was used to profile the metabolites in plasma. GC was used to investigate essential oil components (**Paper I**), and plasma metabolites (Paper VI). An SFC method was developed and optimized (Paper II) and used to profile lipids (Papers III and IV). Since HPLC and GC were only applied as analytical tools in this thesis, the main discussion in this chapter will be focused on method development in SFC.

Lipids are a complex group of biomolecules with diverse structural and functional activities. They have several functions in the body; serving as fuel molecules and energy storage, and are involved in cell signalling and cell–cell interactions and disease states¹⁰¹. Lipids are classified into eight main groups, namely: free fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides⁹. Lipid analysis is required for routine and research analysis in food, pharmaceutical and clinical applications.

Lipid analysis can be carried out with a number of chromatographic techniques. Thin layer chromatography, reversed- and normal-phase liquid chromatography, GC and capillary electrophoresis have been used to profile and quantify lipid species¹⁰². Each technique has drawbacks that limit their application. For example, reversed-phase liquid chromatography provides separation based on acyl chain length and degree of unsaturation, whereas normal-phase liquid chromatography provides separation based on the polar head groups of lipid classes, and the solvents

used as mobile phase are not compatible with MS. Hydrophilic interaction liquid chromatography has been applied for lipid analysis as an alternative to normal-phase liquid chromatography as it is more compatible with MS. However, it also has some limitations in the separation of lipids with similar hydrophobic groups such as cholesteryl esters, triacylglycerols and diacylglycerols.¹⁰³

SFC has become fairly common in separation science. Klesper was the first to describe the use of a supercritical fluid for separation in 1962^{104} . The technique was developed during the 1980s and 1990s from open tubular capillary columns to packed columns¹⁰⁵. The development of packed columns allowed the use of organic modifiers at high ratio to the ScCO₂ in the mobile phase, and that broadening the range of applications to more polar compounds¹⁰⁶. The development of BPRs improved the performance of SFC by allowing the pressure in the system to be more carefully controlled, reducing the variation in density of the mobile phase, compared to a capillary restrictor. Developments in SFC (robustness and high-pressure pump) have made SFC systems compatible with columns that have particle sizes below 2 μ m. The low viscosity of ScCO₂ used as the mobile phase provides faster diffusion than conventional liquid chromatography. Grand-Guillaume Perrenoud et al. investigated the performance of UHPSFC and UHPLC using columns packed with particles of different sizes¹⁰⁷. The separation efficiency (plate height) of a chromatographic method is described by the van Deemter equation:

$$H = A + B/u + Cu$$

where A is a term related to multiple flow paths, B is the longitudinal diffusion term, and C is the mass transfer term. Figure 14 shows the van Deemter curves obtained for 3.5 and 1.7 μ m particle size columns using UHPLC and UHPSFC. In general, the separation efficiency of both HPLC and SFC decreases with decreasing particle size. Also, SFC and HPLC have the same minimum plate height for the same particle size, but the optimal linear velocity for UHPSFC is approximately five times higher than for UHPLC. This is because the C term is proportional to the ratio of the square of particle size and the diffusion coefficient, and since the diffusion is high in SFC, running at high flow rates has only a minor influence on the plate height¹⁰⁷.

The density of the mobile phase can be varied by varying the temperature and pressure (as mentioned above) and this may change the elution strength. Neat CO_2 has solubility properties closed to those of *n*-hexane, which limits the application of SFC to specific classes of compounds. The application of SFC can be extended by mixing polar organic solvents such as methanol with the ScCO₂ to solubilize more polar compounds. SFC thus has the ability to efficiently and selectively resolve a wide range of lipid classes with different polarities¹⁰⁸.

Paper II describes the screening of seven sub-2 μ m columns for lipid profiling using SFC. A new separation method was developed, SFC-QTOF, based on the best

column for global lipid profiling. The method developed was applied to profiling lipid species from Moringa oil (**Paper III**) and various berry seeds (**paper IV**).



Figure 14. Van Deemter curves for butylparaben using 2 systems equipped with columns containing 1.7 or 3.5 μ m particles. XTerra RP₁₈ 50 mm × 4.6 mm, 3.5 μ m, Acquity Shield C₁₈ 50 mm × 2.1 mm, 1.7 μ m both columns tested under liquid chromatography conditions. Acquity UPC² BEH 2-EP 100 mm × 3.0 mm, 3.5 μ m and 100 mm × 3.0 mm, 1.7 μ m columns tested under SFC conditions: (with permission from Ref. ¹⁰⁷).

5.1 Method development in SFC

A fundamental understanding of the chromatographic technique and knowledge of the properties of the sample will simplify the development of chromatographic methods. In this section, the steps necessary in the systematic development of the SFC method for lipid profiling will be discussed.

5.1.1 Sample/analyte properties

The physical and chemical properties of the sample and analyte, including the chemical structure of the target compounds, their solubility, pKa, concentration range, UV-vis activity, and method of ionization, should be known/must be determined. The solubility of the sample in the SFC mobile phase is very important. Compounds that are soluble in water (i.e., highly polar) might not be separated using SFC. There is also a risk when using pure water as a modifier in SFC as the solubility of water in CO_2 is very poor, which could lead to phase separation. Phase separation can alter the response of the detector and reduce the repeatability of the method. A mixture of water and methanol can be used as a modifier in SFC, allowing polar compounds to be separated in a manner similar to hydrophilic interaction liquid

chromatography. As lipids are soluble in ScCO₂ with a methanol modifier, SFC was deemed to be a good starting point for development (**Paper II**). The complexity of the sample must also be considered and the kind of interference that can be expected, in order to adapt the method so as to be more selective for the target compounds. Method development was initially undertaken with real plasma extract, and the exact m/z ratio was used to identify the species (**Paper II**). However, some species from different lipid classes have the same m/z ratio; for example, PE 35:1 and PC 32:1 both have m/z = 732.5538 in form of $[M+H]^+$. Therefore, known lipid extracts (commercial standards) were thereafter used to spike plasma extracts in order to identify the lipid classes/ and species and helped to solve this problem.

5.1.2 Choice of mobile phase

Carbon dioxide is the main component of the mobile phase in SFC. As mentioned above, a modifier is added to increase the polarity of the mobile phase so that more polar analytes can be separated. The choice of mobile phase composition can also affect the interactions between the analytes and stationary phase (i.e., change the selectivity). West et al. investigated the effects of different modifiers (methanol, ethanol, isopropanol and acetonitrile) on the retention time and selectivity of a variety of drug compounds¹⁰⁹. The use of acetonitrile led to a different selectivity than the other modifiers, particularly for basic compounds, and the authors attributed this to the poorer ability of acetonitrile to cover the free silanol groups.

Another factor that must be considered when choosing a modifier is the solubility of the additive (salts) in the modifier, as the additives are added to the modifier, not the CO₂. In the study described in **Paper II**, the addition of salt was essential to improve the separation of the lipids. Therefore, acetonitrile was excluded because of the limited solubility of ammonium salts. In addition, the poor ability of acetonitrile to cover the free silanol groups could influence the peak shape, as polar lipids may interact strongly with free silanol groups. Acetonitrile has a lower solvating power than methanol, which could increase the retention time of polar lipids. For these reasons methanol was chosen as the mobile phase modifier.

5.1.3 Additives

Additives such as organic bases, organic acids and salts, are added to the mobile phase for several reasons, for example, to adjust the pH of the mobile phase. This is more *pronounced* in liquid chromatography. In SFC, the pH of the mobile phase with methanol has been found to be around 5, and the addition of strong bases had little effect on the overall pH¹¹⁰. Strong acids can reduce the pH in SFC to below 1.7. Additives can also be used to improve the peak shape of polar compounds, by the formation of ion pairing between the additive and the analyte, which can affect the electrostatic interactions between the analyte and the stationary phase. The

additive may also interact with the active silanol group in the stationary phase, and avoid secondary retention¹¹¹. An additive can be used to improve the ionization and detectability of the ions in MS¹¹². In SFC, the additive can be added via the make-up solvent, provided it has no impact on the separation efficiency. For example, Jumaah et al. added ammonium formate to the make-up solvent to enhance the ionization and adduct formation in MS¹¹³. The absence of an additive in the mobile phase in the study described in **Paper II** caused broadening of the phospholipid peaks due to the very strong interaction of the polar head group with the free silanol groups in the stationary phase. Ammonium formate was found to give a similar improvement in the peak shape to ammonium acetate, however, ammonium acetate causes more ion suppression than ammonium formate, which may be due to the higher volatility of ammonium formate.

5.1.4 Stationary phase

In principle, HPLC columns can also be used in SFC, but due to the increased interest in SFC, new columns have been introduced specifically for SFC. Both reversed-phase and normal-phase columns can be used in SFC. The chemistry of the stationary phase has the greatest influence on the selectivity and separation efficiency. West et al. used a solvation parameter model (electron lone pair (E), dipole-type (S), hydrogen bond acceptor (A), hydrogen bond donor (B) and dispersion (V)) to characterize columns according to their interactions¹¹⁴. As mentioned above, due to the complexity and diversity of lipid classes and species, all kinds of interactions can be expected with lipid species. Ashraf-Khorassani et al. reported high-strength silica C_{18} SB to be the best column, showing the highest selectivity for free fatty acids using SFC¹¹⁵. The ethylene-bridged hybrid (BEH) 2-EP column has been found to provide the highest resolution when separating triacylglycerols and diacylglycerols in SFC¹¹⁶. Therefore, considering the many possible interactions between lipid species and the stationary phase, there is no way of predicting which will be the most suitable column. A screening approach was adopted in the present work to assess the performance of seven stationary phases with different chemistries (Figure 15). In such an approach, the target analytes must be defined, and should represent different lipid classes. The response(s) used to evaluate performance should also be carefully chosen and defined. It may be useful to use a generic chromatographic method in the screening stage to investigate the behaviour of each column. The results obtained can then be used to determine the way forward. Seven columns, 54 lipid species and 4 responses were used in the study presented in Paper II, making manual data analysis difficult. Therefore, a multivariate analysis approach, i.e., principal component analysis, was used, as discussed in the next chapter. The results showed that Diol column provided the highest separation resolution within and between lipid classes. It also gave peaks with small peak widths in a reasonable run time.



Figure 15: Chemistries of the stationary phases in the columns investigated (**Paper II**). 2-PIC: 2picolylamine; CSH-FP: charged surface hybrid fluoro-phenyl; HSS-C₁₈: high-strength silica C18 SB; DEA: diethylamine; 1-AA: 1-aminoanthracene; Diol: high-density diol; and BEH: ethylene bridged hybrid. (Figure reprinted from Waters application notes with permission.)

5.1.5 Injection solvent

Ideally, the injection solvent should have a composition similar to the initial composition of the mobile phase, as this increases the concentration of the injection plug, which should result in sharper peaks. In other words, concentrating effect will increase when the elution strength of the injection solvent is less than the elution strength of the initial composition of the mobile phase. Thus, the properties of the solvent used to prepare samples for SFC can affect the peak shapes. Abrahamsson et al. investigated the impact of injection solvent properties on the chromatographic efficiency (plate numbers)¹¹⁷ of SFC using polar and nonpolar columns. The interactions between the injection solvent, the analyte and the stationary phase affect the retention behaviour and the peak shapes. However, it is also very important to consider the solubility of the analytes in the injection solvent. In the present work, the chromatographic efficiency was improved when the lipid extract was redissolved in a mixture of isopropanol, acetonitrile and chloroform (1:1:1, v:v:v), compared to methanol:chloroform (1:1, v:v) (**Paper II**). This was attributed to the lower elution strength of the isopropanol, acetonitrile and chloroform mixture. However, a mixture of isopropanol, acetonitrile and chloroform (1:1:1, v:v:v) was found to have a lower ability to dissolve plasma lipid extract than methanol:chloroform (1:1, v:v). Decreasing the ratio of methanol to chloroform from 1:1 to 1:2 (v:v) improved the peak shapes without reducing the solubility, as the Diol stationary phase and methanol can interact through dipolar and hydrogen bonding.

5.1.6 Optimization of chromatographic conditions

The effects of column temperature, outlet pressure and flow rate were investigated using a design of experiments (DoE) approach (Paper II). The compressibility and density of the mobile phase is mainly affected by these parameters, and they will therefore affect the chromatographic performance. The impact of these parameters is more pronounced at the beginning of the gradient since the composition of the mobile phase is neat CO₂, or CO₂ with a very low modifier content. For example, high column outlet back-pressure increases the density of the mobile phase, which increases the solvating power, thus reducing the retention of early eluting lipids. However, late eluting lipids are less affected as they require a high percentage of non-compressible modifier to elute. Increasing the column temperature has the opposite effect on the retention. Increasing the temperature initially decreases the density of the mobile phase (low elution strength), which leads to a long retention time. Low elution strength increases the resolution of early eluting lipids. Increasing the flow rate can increase the pressure at the inlet of the column and thus increase the density of the mobile phase, particularly at the beginning of the gradient. In addition, peak deterioration might occur for early eluting peaks due to the pressure drop caused by the expansion of CO_2 gas at the outlet of the column¹¹⁸. This effect can be decreased by introducing the make-up solvent.

5.1.7 Detector

The most common detector used in lipid profiling (also called lipidomics) is a mass spectrometer. Several analysers are available, which differ in their sensitivity, selectivity and mass resolution¹¹⁹. One of the goals of method development is to identify the most appropriate MS analyser for each case. Identification of the ionization technique is also important to increase the sensitivity of the method. For instance, vitamin D and its metabolites are more easily ionized by atmospheric pressure chemical ionization than electrospray ionization (ESI)¹²⁰. Direct infusion can be used in MS without being coupled to a chromatographic technique. This method is rapid and inexpensive, but suffers from low selectivity, low sensitivity due to ion suppression, and identification is not easy¹²¹. The main aim of developing the SFC method described in Paper II was to profile lipid species in different samples. A mass analyser with high mass resolution that enables identification based on the exact m/z ratio is more suitable for untargeted analysis. QTOF-MS offers fast scanning with high mass resolution for identification. ESI is the most common ionization technique for lipid analysis. The major lipid classes can be easily ionized either in positive or negative mode.

If the aim is to quantify the lipid species in a sample or compare lipid abundance in different samples, an internal standard is needed to correct for variation in the chromatographic or MS equipment¹²². Variation in the response can be caused by variations in the chromatographic operating conditions, the sample matrix or ionization efficiency. Internal standards should have similar properties to the targeted analytes, but should not be present in the sample. Isotopically labelled standards are common in lipidomics, but they are costly. In the present work, internal standards were used to correct for the variation in the peak in order to compare the response from different sample groups (**Papers I** and **IV**). In the study presented in **Paper IV**, isotopically labelled standards representing different classes of lipid classes were used to compare their abundance in different berry seeds. In the study described in **Paper VI**, three different compounds covering a wide range of polarities were used since plasma metabolites may include analytes with different properties.

The operating conditions for the ion source in MS are dependent on the analyte and the composition of the mobile phase. It is difficult to find optimal operating conditions for all lipid classes in SFC since the composition of the mobile phase varies considerably. Furthermore, the structure of the lipids varies from hydrocarbons, such as cholesterol ester, to lipids containing polar lipids, such as phospholipids. In the study described in **Paper II**, a statistical method based on design of experiments was used to find the best conditions throughout the chromatographic run. The results showed that desolvation drying gas (nitrogen) temperature and flow rate have opposing effects on the ionization process for early and late eluting analytes, due to the composition of the mobile phase (Figure 16). At an early stage of the elution, CO_2 is the main component of the mobile phase, and a low temperature and low flow rate are sufficient to evaporate the solvent during ionization, while at the end of the elution the situation is the opposite, and higher temperature and flow rate of the gas are need.



Figure 16. Coefficient plots showing the influence of MS parameter settings on the peak area for the early and late eluting lipids CE 18:3 and PE 38:4, respectively. Cap. V: capillary voltage; Cone V: cone voltage; Des. T, desolvation gas temperature; Des. F: desolvation gas flow rate.

QTOF-MS is a tandem MS (MS/MS) technique that can be operated in different acquisition modes. The aim of the study and complexity of the sample matrix determine the operating mode during data acquisition. In a full mass scan, the ions formed in the ion source arrive at the TOF analyser without being fragmented, unless from ionization process. This mode gives the exact m/z ratio, providing information on the different analytes present in the sample. It can be used for quantification, but the information provided in this mode not sufficient for identification. The second acquisition mode is the data-independent acquisition (DIA) mode, and it is denoted MS^E in Waters OTOF. In this mode, the ions formed in the ion source are transported to a collision cell through the quadrupole. The voltage in the collision cell is ramped between low and high voltage. Ion fragmentation occurs only at the higher voltage. This mode provides MS and MS/MS data in a single run¹³². However, is it difficult to match each parent ion to its fragments in the case of coeluted peaks. This acquisition mode was used in the studies presented in Papers II, III and IV. The third acquisition mode is the data-dependent acquisition mode, which is more selective than the DIA mode. It is based on the generation of a precursor ion list from the full first scan cycle, which is applied to targeted fragmentation in the second scan cycle¹²³. This approach has been used to enhance the range of MS/MS for metabolite identification^{124,125}. This method was applied in the study presented in **Paper VI** to identify the metabolites in plasma. In the first cycle, all the ions are transported to the TOF analyser, while a subset of the most intense ions is selected for MS/MS in the second cycle. In this cycle, quadrupole filters select ions one by one and the collision cell is operated at high voltage to induce fragmentation. The identities of the compounds can then be determined from the MS/MS data. This method provides more precise data for identification, although there may be co-elution of several analytes. Targeted MS/MS with a preset precursor list can also be performed in QTOF-MS. In this approach the quadrupole filters the ions according to the preset precursors which are then subjected to a high voltage in the collision cell, and all the fragment are scanned in the TOF analyser.

6 Chemometric tools and data processing

The rapid developments in extraction, separation and detection techniques in analytical chemistry have led to the generation of huge amounts data which cannot easily be analysed manually. For example, data produced by MS and spectroscopic techniques are very complex and difficult to resolve or interpret manually. Tools are therefore required to design experiments, optimize the analysis and interpret and understand the results. The field of chemometrics was therefore developed to facilitate the performance of complicated experiments and complex data analysis. D. L. Massart defined chemometrics as "the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analyzing chemical data"¹²⁶. Many chemometric tools were used in the present work to understand the effects of the operating parameters in extraction, chromatography and MS, and to identify the optimal operating conditions. Chemometric tools were also used to visualize, classify and interpret the data.

6.1 Design of experiments

In order to obtain maximum performance with any analytical technique, the optimal operating conditions must be used. The optimal conditions can be found by manually varying one parameter at a time, while keeping the others constant. However, this would require many experiments, the information obtained would reflect the conditions at those points only, and the interaction between factors is not taken into consideration¹²⁷. It is thus better to use a DoE approach. DoE is defined as "a rational and cost-effective approach to practical experimentation that allows the effect of variables to be assessed using only the minimum of resources"¹²⁸. In this approach, a series of experiments, representative of the investigated range of operation of the process, is performed. A reference point (centre point) is defined, and experiments are then conducted around this point. DoE was used in the present work to screen and optimize the extraction process (**Papers I, III** and **IV**), and chromatographic and MS parameters (**Paper II**).

The first step in DoE is screening, in which the factors that have significant influence on the response are identified, and the suitable operation range determined. Excluding insignificant factors reduces the risk of model over-fitting, and thus increases prediction reliability. For screening purposes, the assessment of two levels of each factor is suitable, as the objective is to understand the behaviour of the process, rather than understand the process. The exact choice of factor and the range are sometimes determined by the physical constraints of the equipment. for example, temperature or pressure limits. Determination of the significant factors in the screening stage is followed by optimization, in which a few factors are investigated in many experiments. Optimization aims to study the impact of factors and their interactions on the response. Also, it aims to find the condition where all factors give the optimum response. Many designs can be used to create a DOE model. The choice of design depends on the objective of the study, the type of factors (qualitative or quantitative), the constraints and the number of factors to be investigated. Two designs were used in the present work namely, the face-centred central composite design (FC-CCD) and the Box-Behnken design (BBD) (Figure 17). Both these designs are common in the optimization of 2-4 quantitative factors.



Figure 17. (A) Face-centred central composite design (FC-CCD) and (B) Box-Behnken design (BBD)¹²⁹.

Both FC-CCD and BBD are quadratic designs, which means that the relation between the factors can be expressed as a curve. These designs are therefore common in response surface methodology. BBDs usually have fewer design points than FC-CCDs, and are therefore less expensive to run with the same number of factors. Furthermore, the BBD does not include runs where all the factors are at their extreme settings, for example, all at the lowest settings¹³⁰.

Randomization of the order in which the experiments is run is extremely important to avoid bias in the results, and to establish a reliable base for the estimation of errors in the design¹³¹. The data obtained using DoE are used to estimate the coefficients of the model. The coefficients can be estimated by multiple linear regression or

partial least squares (PLS). Multiple linear regression is used when there is one or only a few responses, while PLS is used when there are many responses in the design¹²⁸. When fitting the model, the most important diagnostic tool consists of the two parameters R^2 and Q^2 . R^2 is a measure of how well the regression model can be made to fit the raw data and it is called goodness of fit. Q^2 estimates the predictive power of the model and it is called goodness of prediction. The effect of the factors on the response can be visualized by plotting the coefficients (Figure 16). Normally, the first coefficients describe the linear effect of each factor. Each coefficient indicates the change in the response when the value of a factor is increased from the centre point to its highest level, while the other factors are kept fixed at their centre points. Error bars represent the magnitude of the variation between the dat. The coefficient has error bar cross the original means the factor does not have significant influence on the response. The interaction coefficients indicate whether there are interactions between the factors. Response surfaces and contour plots can be used to visualize the interactions between different combinations of factors and their influence on the response. Careful study of different combinations of the factors can provide an indication of the range of optimal conditions. In some cases, it is difficult to identify the optimal conditions due to the opposing effects of factors, or due to the presence of many responses that have different criteria (maximum, minimum or targeted to a value). In such cases, various tools are available to help identify the optimum, for example, the simplex optimization function.

6.2 Data processing

It is often necessary to pre-process MS data to generate a practical data matrix. The reason for this is to eliminate noise and any potential bias in the data, and to reduce the complexity¹³². Many software tools have been developed to process raw MS data acquired with a chromatographic system coupled to MS. Common functions in these tools include noise filtering and baseline correction, peak detection and deconvolution, alignment and normalization¹³³. Peak picking may be untargeted as described in **Paper VI**, or targeted as in **Papers II-IV**. Targeted data processing requires prior knowledge concerning the analytes in the sample, for example, the retention time and exact m/z. Untargeted data processing requires the definition of a signal threshold (differentiate from noise) and a range of m/z. The definition of the threshold between noise and a signal is important, especially in the case of peaks with low intensity. In addition, the tolerance in m/z and retention time are important in the alignment process. The results of data pre-processing can be selected among different chromatographic criteria such as m/z, peak area, peak height, retention time, peak width and peak duration.

6.3 Data exploration

Studies related to metabolomics or screening for multiple responses produce enormous amounts of data that cannot be analysed manually. For example, in the study presented in **Paper VI** a matrix of 2000 x 30 (mass features/signals x number of samples) was generated, and in the study described in Paper II a matrix of 270 x 21 was obtained from MS analysis. Projection methods provide a very intuitive and visual approach for data analysis. These methods are useful in resolving multivariate data by reducing their dimensionality into a number of uncorrelated latent variables¹³⁴. A latent variable is a variable that is not directly observable, but is assumed to affect the response variables. Principal component analysis (PCA) is a well-established technique that has been used in multivariate data analysis. PCA transfers the data from the original space to a new space to reduce the original multidimensional space into a space of lower dimensionality by finding a linear combination between the data, which is called a component¹³⁵. PCA is concerned with the visualization of latent data structures using graphical plots. The interpretation in PCA is based on all variables simultaneously, instead of individually, which allows a deep understanding of the relation between the variables. PCA is generated based on the direction in the data which demonstrate the highest variation. PCA can be represented as scores or leading plots. The scores of the principal components are the weighted sums of original variables, and the weights are called loadings. The scores contain information about the objects (samples), and the loadings of the variables. Similar objects are grouped together in the score plot. Detection of outliers is also possible in PCA since they do not belong to a certain class of sample. Figure 18 shows the score plot of the MS data obtained from different extracts of Moringa seed oil using ScCO₂ at two pressures, 40 and 80 MPa, with and without a modifier (ethanol), for two different Moringa species, Moringa oleifera and Moringa peregrina. Interestingly, the first component discriminates Moringa peregrina samples from Moringa oleifera, whereas the second component reveals the impact of adding a modifier to ScCO₂.



Figure 18. PCA score plot of oil samples from two Moringa species obtained under different SFE conditions (from Paper III). MO *Moringa oleifera;* MO *Moringa peregrina*

PCA is designed to describe the largest variation in the data, without considering the class to which they belong. Hence, other tools such as PLS may be more powerful when comparing two classes. PLS is used to analyse and predict a set of dependent variables (Y) from a set of independent variables (X). Orthogonal partial least-squares analysis (OPLS) is a modification of the PLS regression analysis method. It can separate the systematic variation in the variable X into two parts, one with a linear relationship to Y, and the second which is orthogonal to Y¹³⁶. Discrimination analysis of OPLS (OPLS-DA) is a version of OPLS used when there are two classes and one wants to exploit the differences between them¹³⁷. The most popular strategy for validating the OPLS-DA model is internal validation based on cross-validation, but it is also recommended to use an external prediction set. Several OPLS models can be calculated using a common reference, and correlations given by the models can be combined in a so-called shared and unique structure plot¹³⁸, as in the study described in **Paper VI**, and shown in Figure 19.

7 Societal impacts of this research

Plant extracts contain a variety of bioactive compounds that can be used as functional food ingredients, or in the production of medicines and cosmetics. Nowadays, more than 80% of food supplements and ingredients and 30% of drugs are produced from bioactive natural products¹³⁹. Cragg et al. found that 30% of the drugs approved by the US Food and Drug Administration between 1983 and 1992 were based on natural products¹⁴⁰. The health impact of bioactive compounds from plants is the driving force for their extraction and analysis. This chapter presents a discussion based on **Paper VI** on how bioactive compounds in the lingonberry can reduce the negative impact of a high-fat diet. Later in this chapter, industrial applications of bioactive compounds are discussed.

7.1 Lingonberry as a food supplement

Berries have gained increased interest in recent years due to their high contents of bioactive compounds, including phenolic compounds, vitamins and omega-3 fatty acids¹⁴¹. Lingonberries grow wild in mountainous and forested areas. They are rich in anthocyanins, proanthocyanidins and resveratrol¹⁴². The lingonberry has several beneficial health effects. Mane et al. reported that feeding rats a diet supplemented with lingonberries reduced their total oxidant status¹⁴³. Heyman-Lindén et al. also found that lingonberries had the ability to prevent obesity and metabolic abnormalities associated with type 2 diabetes¹⁴⁴. However, the content and levels of bioactive compounds may vary among same type of food. Geographical origin, plant nutrition, time of harvest and storage conditions may lead to variations in the phytochemistry of plants¹⁴⁵. Such a variation was observed by Heyman-Lindén et al. when they repeated their experiment with a new batch of lingonberries. The results obtained from phenotype measurements on the two batches of lingonberries were significantly different. The first batch (L1) lowered glucose and body fats % in comparison to the control while the repeated experiment (L2) showed insignificant differences with the control.

In the study presented in **Paper VI** it was found that both batches of lingonberries L1 and L2) had similar compositions regarding phenolic compounds, although the level of some compounds was higher in L2, and the diet containing these berries was less effective in reducing body fat. Profiling of blood metabolites helps to

understand the way in which food components can *alter the phenotypes* and prevent or induce the development of disease¹⁴⁶. In this study it was found that supplementing a high-fat diet with lingonberries improved the liver function, as indicated by improvements in liver mass, high-density lipoproteins and total cholesterol. Metabolite profiling revealed that lingonberry supplementation decreased the levels of sphingomyelins (SMs) and cholesterol, and increased levels of phosphatidylcholines (PCs), lyso-phosphatidylcholines (LPCs), and lysophosphatidylethanolamines (LPEs) in plasma, as can be seen in Figure 19.



Figure. 19. Plot of shared and unique structure (SUS) showing differences in most of the plasma metabolites common to L1 and L2. L1 contained less glucose and more free fatty acids than L2. Serine decreased in both batches compared to high-fat diet¹⁴⁷.

Elevated plasma SM levels have been associated with increased risk of cardiovascular disease¹⁴⁸. It has also been suggested that foods that lead to a reduction in SM levels may prevent cardiovascular disease, diabetes and cancer¹⁴⁹. The findings of the present work suggest that lingonberries alter the synthesis pathway of SMs by decreasing the level of serine (Figure 20). The bioactive compounds in the lingonberry appear to have modulated the metabolic process, preventing the formation of metabolites associated with an unhealthy phenotype

such as SMs by decreasing the level of serine. It was also found in this study that batch-independent results were not caused by phenolic compounds, since both batches had similar compositions and concentrations of phenolic compounds.



Sphingomyelin (+DG)

Plant foods contain a very complex mixture of bioactive compounds, which can alter the metabolism and may improve our health. It is difficult to associate a particular effect to a specific group of compounds. In many cases, the combination of several compounds has a synergistic effect on health. Food intervention studies should always be conducted with several batches of the same food to avoid exaggeration of unique metabolic effects, which although interesting, may not be representative of the investigated food.

7.2 Oil and oil-soluble compounds

Essential oils are extracted commercially from different aroma plants for use in fragrance and cosmetic products. Essential oil from *Boswellia sacra* resin has been used as a therapeutic-grade and perfume ingredient. Since the oil from this resin is rich in α -pinene, it can be used as a starting material for the synthesis of other chemicals¹⁵⁰. Extraction of the oil using ScCO₂ may be more economically feasible than steam distillation¹⁵¹. In addition, the quality and quantity of oil components obtained using SFE are higher than with steam distillation¹⁵², as the high temperature of the steam can cause decomposition and the loss of volatile compounds. In the study described in **Paper I**, it was shown that extraction was faster and higher amounts of α -pinene and cis-verbenol were obtained using CXLE than with SFE and SLE, which will increase the value of the oil and reduce the production cost.

Moringa oil is a valuable product in the food, cosmetic and pharmaceutical industries. In Africa, for example, the oil from *Moringa oleifera* is used as a cooking oil. Mechanical pressing is the most common method of extracting oil from Moringa

Figure 20. Suggested mechanism showing how lowering of the concentration of serine influences the availability and formation of PCs and SMs.

seeds. Del Valle et al. reported that the amount of oil extracted from rosehip was 23% higher when using ScCO₂ extraction at 30 MPa than cold pressing¹⁵³. In addition, the oil obtained using ScCO₂ is solvent-free and subject to less oxidation due to the inertness of the solvent and the low extraction temperature. In the study presented in **Paper III**, it was shown that recovery of oil using UHPSFE was high in a short time.

Microalgae constitute a promising sustainable source of oil-soluble carotenoids. Over 700 carotenoids have been identified in microalgae¹⁵⁴ of which lutein has gained noticeable attention. Lutein has been widely used as a natural colorant in the food industry. It is used for colouring fish and poultry, drugs and cosmetics. In addition, lutein and zeaxanthin are the only carotenoids reported to be present in the retina and lens of the eye¹⁵⁵. However, not all solvents can be used in food and pharmaceutical applications. The Europe Union has approved a list of solvents for use as food additives in the food industry¹⁵⁶. Acetone, methanol, ethanol and isopropanol are examples of approved solvents. In an independent study (ongoing), we are examining the possibility of using SFE and PLE techniques with approved solvents including ethanol and methanol, to extract lutein from microalgae efficiently and economically.

7.3 Utilisation of food waste in a circular economy

Other example of the industrial application of bioactive compounds is the use of industrial press cake by-products as ingredients in high-value food products. The by-product from the berry juice industry, pomace, is rich in bioactive compounds such as polyphenols, fatty acids and dietary fibre¹⁵⁷. Incorporating berry pomace in food products could enhance the nutritional and sensory properties of the products, while increasing the sustainability of the food chain. The physicochemical properties of pomace from different kinds of berries, including blackcurrant, redcurrant, chokeberry, rowanberry and gooseberry, have been characterized in collaboration with researchers at Dresden Technical University¹⁵⁸. In this study, the composition and amounts of phenolic compounds in pomace were analysed and compared. The aim of this study was to investigate the nutritional value of pomace from each kind of berry in order to assess their applicability for use in complex food systems. The method used to dry the pomace could influence the bioactive compounds, as high temperatures may cause degradation of some bioactive compounds. Therefore, the influence of two drying methods on the stability of phenolic compounds was evaluated: an air oven and freeze-drying. The results showed that the phenolic content, particularly the anthocyanins, were retained when freeze-drying was used (manuscript in preparation). The stability of high-value compounds such as phenolic compounds throughout the production chain, from

pomace to final products such as muffins and crackers, is important as it is known that the temperatures used for baking degrade phenolic compounds.

The benefits of incorporating berry pomace into food products are not only associated with nutritional benefits. Such a project will pave the way for the investigation of other by-products that could be of use in added-value products. Pomace will thus become a product rather than a waste which will increase its economic value. This will have benefits for all those involved in the food chain (farmers, suppliers and manufacturers). Improvements in this sector will provide more opportunities for employment and more small companies will be involved. Another aspect of incorporating pomace in food products is that people will become more aware of healthy products, which may lead to a long-term improvement in health.

8 Conclusions and future work

The work described in this thesis has been dedicated to developing and implementing supercritical CO_2 for the study of bioactive compounds from complex samples by combining fundamental knowledge and practical applications. Integration of CO_2 in extraction and chromatographic techniques offers many advantages regarding selectivity, extractability, recovery, resolution and sustainability. The studies presented in **Papers I** and **V** showed that liquid CO_2 could be added to the liquid organic solvent in CXLE to tune the solvent's solubility parameters, thus changing its selectivity. The addition of liquid CO_2 also reduced the viscosity, which improved the mass transfer properties. In the study described in **Paper I**, extraction was found to be faster using CXLE than SFE or SLE. Modelling of the CXLE of solid materials is important in order to understand the extraction kinetics and its fundamentals. In future work, it would be interesting to combine the solubility data for quercetin obtained from CXL with the data reported in **Paper V** in order to develop inverse modelling CXLE solubility.

The findings reported in **Paper IV** showed that combining sonication with CXLE increased the recovery of oil from berry seeds by about three times. Solubility is always an important factor in batch extraction and it is important to consider the solvent-to-sample ratio. It would therefore be worthwhile to investigate the effects of sonication combined with dynamic extraction using CXLE to determine whether this could increase the loading amount of extracted sample.

In the study described in **Paper III**, the use of UHPSFE to extract oil from Moringa seeds was investigated. The results showed that increasing the pressure significantly increased the amount of oil extracted. Ultrahigh pressure also changed the selectivity, as evidenced by the fact that some phospholipids were detected in the oil extracted at 80 MPa. The addition of an organic modifier increased the total amount of oil extracted and enhanced the solubility of polar lipids compared to neat ScCO₂. The dynamic addition of modifier during UHPSFE may increase the extraction and recovery rate.

The aim of the study presented in **Paper II** was to screen many stationary phases for global lipid profiling using UHPSFC. Diol columns offered high resolution within and between lipid classes in a short time. The method could thus be used to profile a wide range of lipid classes. The method was used to profile lipids in oil samples from Moringa seeds and berry seeds. The DIA mode in negative ESI could be used to profile the fatty acids associated with phospholipids and sphingolipids since these classes produce FFAs when fragmented in the collision cell in negative ESI.

The results presented in **Paper VI** suggest that lingonberries have the ability to reduce the negative impact on health of a high-fat diet. The ingestion of lingonberries improved the liver function, and this was suggested to be due to changes in sphingomyelin metabolites. This study also revealed the need to perform such food interventions with several batches of the supplement from different sources. Performing a study large number of different lingonberry batches would allow identification of the bioactive compounds that play an important role in promoting health.

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